Protein Synthesis and Long-Term Memory Formation in the Day-Old Chick

Thesis

Protein Synthesis and Long-Term Memory Formation in the Day-Old Chick

By

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List of Abbreviations

AMP	Adenosine triphosphate
AMPA	γ-amino-3-hydroxy-5-methylisoxazole-4-propionic acid
ANI	Anisomycin
AP-1	Activator protein-1
AVP	D,L-2amino-5-phosphonovalerate
cAMP	Cyclic-AMP
7-CK	7-chlorokynurenine
CaM kinases II	Ca2+/calmodulin-dependent kinase type II
CaRE	Calcium responsive element
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CRE	cAMP responsive element
CREB	CRE-binding protein
DAB	Diaminobenzimine
2-Dgal	2-deoxygalactose
2-DG	2-deoxyglucose
EAA	Excitatory amino acids
ECL	Enhanced chemiluminescence
epsp	Excitatory post-synaptic potential
c-*fos*	Cellular fos mRNA
Fos	Fos protein

GABA	γ-aminobutyric acid
HA	Hyperstriatum accessorium
HP	Hippocampus
IEG	Immediate-early gene
IEGP	Immediate-early gene product
IgG	Immunoglobulin G
IMHV	Intermediate medial hyperstriatum ventrale
i.p.	Intraperitoneal
ITM	Intermediate-term memory
c-*jun*	Cellular jun mRNA
Jun	Jun protein
LPO	Lobus parolfactorius
LTD	Long-term depression
LTM	Long-term memory
LTP	Long-term potentiation
MeA	Methylanthranilate
MK-801	Dizocilpine
mRNA	Messenger RNA
MTZ	Metrazole
NCAM	Neural cell adhesion molecule
NMDA	N-methyl-D-aspartate
NO	nitric oxide
PBS	Phosphate buffered saline

PKA	Protein kinase A
PKC	Protein kinase C
PLA_1	Phospholipase A_1
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PSD	Post-synaptic density
RNA	Ribonucleic acid
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
STM	Short-term memory
STP	Short-term potentiation
TBS	Tris buffered saline
TEMED	Tetramethylethylenediamine

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ABSTRACT

Protein synthesis, and more specifically the immediate-early genes (IEG), Fos and Jun induction was studied after passive avoidance training in the day-old chick.

Polyclonal antibodies directed against Fos and Jun proteins revealed two anti-Fos and two anti-Jun immunoreactive bands with apparent molecular weights of 47, 54, 39 and 54 kDa respectively. Since the 54 kDa band was common to both antibodies it maybe an activator protein -1 complex (AP-1 protein). These bands were expressed in both left and right intermediate medial hyperstriatum ventrale (IMHV) and lobus parafactorius (LPO) up to four hours after training. The MeA training experience significantly induced these bands in the left IMHV 1 hour and right LPO 2 hours after training.

Injection of the NMDA receptor antagonist MK-801 prior to training attenuated learning-associated induction of these proteins at 1 hour in the left and right IMHV and 2 hours in right LPO.

Fos and Jun immunocytochemistry 2 hours post-training showed that activity increased in both left and right IMHV and LPO, with the greatest rise in right LPO. Induction in all areas was abolished by the inhibitor of protein synthesis, anisomycin (ANI).

Studies of the amnestic effect of ANI showed two periods of ANI sensitivity, one when injected up to 90 minutes and the second between 4-5 hours post-training. This second period of ANI sensitivity preceded the glycoprotein synthesis inhibitor sensitive phase by about 1 hour.

CHAPTER 1

Introduction

Memory formation is a basic requirement if an organism is to survive and adapt within its environment. The mechanisms underlying memory formation have fascinated scientists for centuries. It is widely accepted that memory is a feature of neuronal networks. The cellular and molecular bases for memory storage are located at the synapses, and memory formation is assumed to be based on modulation of synaptic efficacy. It is not clear, however, where memory formation, including an integration of information, takes place.

Memory Models

There are many models employed for the study of long-term memory formation (for a full review see Dudai, 1989). However, it is beyond the scope of this thesis to review all the literature. Only aspects of the literature that are important to this thesis are included.

Long-Term Potentiation

Long-term potentiation (LTP) was first described by Bliss and Lømo (1973) in the rabbit. They found that if a high frequency train of stimuli is applied to the perforant pathway of the anaesthetized rabbit then there is an immediate and sustained increase in synaptic strength. Three distinct features of LTP were described: (a) the slope of the excitatory postsynaptic potential (epsp) evoked in the granule cells by perforant path stimulation was immediately increased; (b) the amplitude of the population spike was increased; and (c) the latency of the response was decreased. These changes persisted for hours in the anaesthetized rabbit (Bliss and Lømo, 1973) and for days in the unanaesthetized rabbit (Bliss

and Gardner-Medwin, 1973) or unanaesthetized rat (Douglas and Goddard, 1975). This phenomenon is not exclusive to the mammalian brain or hippocampus (Baranyi and Szente, 1987; Teyler and DiScenna, 1987; Artola et al., 1990) but has been evoked in chick hippocampus (Bradley et al., 1993) and in lizard cerebral cortex (Larson and Lynch, 1985).

LTP is considered by some to be a good model for memory formation because it can be produced in the hippocampus, a structure considered to be important in memory formation and retention (Milner, 1968; 1972; Scoville and Milner, 1957). Richard Morris's group at Edinburgh have attempted with some success, to relate LTP in the rat with learning and memory for the water maze. They demonstrated that blockade of the N-methyl-D-asparate (NMDA) receptor (important in memory formation; see later) by AP5 not only results in impairment of place learning but also prevents the induction of hippocampal LTP (Morris et al., 1986). In addition, the biochemical and morphological changes seen following the induction of LTP have since been described in animal models of memory.

If a brief train of stimulation is applied, LTP is induced with a bursting pattern that is very similar to that seen in many cortical and hippocampal neurones (Douglas, 1977). It is also specific to those neurones which received the LTP stimulus since no neighbouring ("unstimulated") neurones show LTP (eg. see Anderson et al., 1980). LTP shows cooperativity in that it has an intensity threshold, below which only a brief post-tetanic potentiation may develop (McNaughton et al., 1978). Above this threshold, potentiation is a function of the number of fibres activated in the pathway (McNaughton et al., 1978; Bliss and Lømo, 1973). LTP is an associative process, that is two separate yet converging pathways can associate to establish and/or increase LTP in one of these pathways. So, when tetani are delivered simultaneously to both a weak input which cannot sustain LTP on its own and to a strong input that

can, then both inputs can sustain LTP (for a review see Brown et al., 1990). Associativity may provide a synaptic model for classical conditioning, where the weak pathway has been identified with the conditioning stimulus, and the strong pathway with the unconditioned stimulus (see Bliss and Lynch, 1988). Hence, LTP appears to fit the Hebb model (1949) which postulated that an increase in the strength of synaptic connections was required to lay down new memories.

A model of the cascade of events leading to LTP-related synaptic strengthening has been suggested. In this model LTP is divided into two phases: induction and maintenance. There is much debate as to whether these phases occur pre- and/or post- synaptically. Induction is currently believed to occur postsynaptically whereas maintenance is believed to be both pre- and post-synaptic. Calcium is known to be important in the induction phase because no sustained LTP is observed when the concentration of Ca^{2+} is low or absent in tissue slice preparations (eg. Dunwiddie and Lynch, 1979). Conversely, if Ca^{2+} is present in high concentrations, then a form of long-lasting potentiation is observed both *in vitro* and *in vivo* (eg. Grove and Teyler, 1990; Bliss et al., 1984). In addition, injection of chelators of Ca^{2+} block LTP indicating an absolute requirement for Ca^{2+} in the induction of LTP (Lynch et al., 1983; Malenka et al., 1988). The pre- and post- synaptic debate is further complicated by evidence suggesting that tetanus-induced potentiation proceeds in stages. The process begins with a protein kinase-independent phase (short-term potentiation; STP), lasting less than 1 hour, followed by three stages of LTP (LTP_1,-LTP_3), requiring protein phosphorylation, protein synthesis from existing mRNAs, and gene transcription, respectively. The expression of synaptic potentiation probably involves both pre- and post- synaptic mechanisms, not necessarily in the same proportion at each stage. One leads to an increase in transmitter release and the other to changes in the properties of the ion channels which mediate synaptic transmission (for a more detailed

review see Bliss and Collingridge, 1993). Different groups using slightly different experimental procedures for induction of LTP could be achieving different stages of LTP (LTP_1 to LTP_3, or even long-lasting LTP), hence the contradictory results reported.

Protein kinase C (PKC) has also been shown to be important in the induction of LTP. Stimulation of PKC by phorbol esters (Malenka et al., 1986; Malinow et al., 1988; Muller et al., 1988) or direct injection of PKC (Hu et al., 1987) induces an LTP-like potentiation in CA1 of hippocampal slices. Conversely, induction of LTP has been blocked by inhibitors of PKC both *in vitro* and *in vivo* (eg. Colley et al 1990; Asztely et al., 1990). Although these data suggest a role for PKC in the induction of LTP, the relatively short-lived potentiation induced by phorbol esters (30-45 minutes) distinguishes PKC-induced potentiation from classical LTP. Another class of protein kinases, the type II Ca^{2+}/Calmodulin-dependent kinases (CaM kinases II), have also been implicated in the induction of LTP, since inhibition of CaM kinases II blocks the induction of LTP (Malenka et al., 1989; Malinow et al., 1989).

Activation of N-methyl-D-asparate (NMDA) receptors for LTP induction has been shown to occur. At rest the NMDA receptor is blocked by normal extracellular concentrations of Mg^{2+} (Mayer et al., 1984). Depolarisation reduces the affinity of the ion channel for Mg^{2+} and removes this blockade. Binding of glutamate to this receptor coupled with strong depolarization, leads to opening of the ion channel allowing influx of calcium into the postsynaptic cell (Ascher and Nowak, 1986). A relationship between the density of NMDA receptors, and inhibition of LTP induction by the NMDA antagonist APV has been demonstrated, i.e. APV does not inhibit LTP induction in areas that are known to have few NMDA receptors (Wigstrom and Gustafsson, 1984; Kauer and Nicoll, 1988; Errington et al., 1987; Monaghan and Cotman, 1985). In fact, the direct application of NMDA can elicit LTP-like effects which can subsequently

be inhibited by APV (Kauer et al., 1988). It has been reported that there is a non-NMDA component of LTP, which is blocked by antagonists of voltage-dependent calcium channels (Grover and Teyler, 1990). Furthermore it has been demonstrated that the influx of calcium through NMDA-associated channels is not sufficient to increase the intracellular calcium to the required concentration for induction of LTP. These workers have suggested that activation of voltage-dependent calcium channels, or a reduction in the efficacy of Na^+/Ca^{2+} exchange, contributes to the increased postsynaptic calcium during induction of LTP (Heinemann et al., 1990). More recently Collingridge et al (1993) have shown Ca^{2+} entry through NMDA receptors can stimulate Ca^{2+}-induced calcium release from intracellular stores.

Activation of the NMDA receptor in LTP in the rat CA1 region of the hippocampus causes an increase in cAMP levels. It is thought that this increase is due to the NMDA receptor being coupled to adenylyl cyclase via Ca^{2+}/calmodulin (Chetkovich and Sweatt, 1993). In their model of LTP, Chetkovich and Sweatt (1993) argue that this increase in cAMP goes on to activate PKA. This in turn increases the responsitivity of the AMPA/kainate receptor, causing large postsynaptic depolarization with synaptic transmission, leading to an increased probability of induction of LTP. Similarly, this increase in cAMP could also play a role in short-term potentiation (STP), by leading to an increased sensitivity of the AMPA/kiainate receptor.

During the maintenance phase of LTP it has been shown that endogenous glutamate release is required (Bliss et al., 1986), which is blocked if the induction of LTP is inhibited by APV (Errington et al., 1987). However Aniksztejn et al (1989) failed to replicate this glutamate-LTP relationship. Transmitter release requires the presence of Ca^{2+} (Katz and Miledi, 1965) and is directly related to intracellular Ca^{2+} concentrations (Verhage et al., 1989). An increase in the transport of $^{45}Ca^{2+}$ into hippocampal slices following the

induction of LTP has been demonstrated (Baimbridge and Miller, 1981). Subsequent studies showed that these changes occur presynaptically (Kuhnt et al., 1985).

PKC also plays a role in the maintenance of LTP, possibly via stimulation of neurotransmitter release. The PKC substrate B-50 (also known as F_1, GAP-43, or neuromodulin) is located presynaptically (Rosenthal et al., 1987), and phosphorylated following the induction of LTP (Routtenberg, 1985). Phosphorylation of B-50 is a calcium-dependent process which is blocked by inhibitors of PKC (Dekker et al., 1989) but paralleled by changes in transmitter release evoked by depolarization and/or phorbol esters (DeGraan et al., 1988; Dekker et al., 1990). Following the induction of LTP, PKC is translocated from the membrane to the cytosol (Akers et al., 1986). This is paralleled by increased phosphorylation of B-50 *in vitro,* following tetanization *in vivo* (Routtenberg, 1985; Lovinger et al., 1986) and *in vitro* (Schrama et al., 1986). An increase in presynaptic Ca^{2+} concentration and PKC activation has been demonstrated following LTP. This is thought to be important in the maintenance of LTP by enhancing transmitter release (Clements et al., 1990).

CaM kinase II has also been implicated in the later stages of LTP (Reymann et al., 1988). It is found in high concentrations in the post-synaptic densities (Kelly et al 1984). However, inhibitors of CaM kinase II do not completely block the induction of LTP (Malenka et al., 1989; Malinow et al., 1989). There is an increase in the amount of membrane bound calmodulin following LTP induction, with a compensatory decrease in cytosolic calmodulin; 30 minutes later this is reversed (Popov et al., 1988).

Non-NMDA as well as NMDA receptors also appear to be involved in the maintenance of LTP since sensitivity to iontophoretically applied quisqualate gradually increases following the induction of LTP in slice preparations.

Inhibition of the non-NMDA receptor by 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) blocks this increase (Davies et al., 1989). In addition Bashir et al (1991) have demonstrated that a pharmacologically-isolated NMDA receptor-mediated response will sustain robust synapse-specific LTP.

In order to sustain neuronal activity, it is thought that a retrograde secondary messenger is released once the postsynaptic neurone is activated. This messenger then travels back across the synaptic cleft to stimulate further neurotransmitter release from the presynaptic neurone. It has been proposed that arachidonic acid may function as a retrograde messenger in LTP, being released postsynaptically, facilitating further release of transmitter presynaptically (Clements et al., 1991). Arachidonic acid is released by the action of the enzymes phospholipase A_1 (PLA_1) phospholipase A_2 (PLA_2) or phospholipase C (PLC) on membrane-bound phospholipids. PLC causes the phospholipids (phophatidylcholine or phosphatidylinositol) to be converted to diacylglycerol (DAG), which in turn is cleaved to produce arachidonic acid by the action of DAG-lipase. The action of PLA_2 on all subtypes of phospholipids directly forms arachidonic acid. Clements et al (1991) showed that arachidonic acid is initially liberated by the action of PLA_2 (2.5 minutes after the induction of LTP) and later by the action of PLC (after 45 minutes). This is consistent with the the fact that an inhibitor of PLA_2 activity, nordihydroguaiaruetic acid (NDGA), blocks induction of LTP *in vivo* (Lynch et al., 1989). It is thought that PLA_2 is only active during the induction phase of LTP (Massicotte et al., 1990). Since the PLC pathway forms the intermediate DAG, which can also activate PKC, this route may serve a dual function later on in the LTP cascade. There is also evidence that free arachidonic acid is a physiological activator of cytosolic PKC (Khan et al., 1992).

Nitric oxide (NO) is another putative retrograde messenger candidate. NO is derived from arginine in a reaction catalysed by NO synthase, and inhibitors of

this enzyme have been reported by some groups (and not others) to block the induction of LTP (Bohme et al., 1991; Schuman and Madison, 1991). Haemaglobin, a scavenger of NO which is presumably confined to the extracellular space, also blocks the induction of LTP, implying that NO (or another haem-binding molecule, such as CO) is released into the extracellular compartment. In addition, NO increases the frequency of miniature epsps in hippocampal slices (O'Dell et al., 1991). However, NO on its own has not been shown to induce LTP but it may increase transmitter release (O'Dell et al., 1991).

Morphologically a number of synaptic changes have been observed as a consequence of LTP. These include a long-lasting change in the mean area of dendritic spines in the distal two-thirds of the molecular layer of the dentate gyrus (Van Harreveld and Fifková, 1975; Fifková and Van Harreveld, 1977). This was attributed to a reduction in the length of the spine neck and a swelling in the spine head (Fifková and Anderson, 1981). Consistent with this was the observation that the number of concave spine heads in the medial layer increased following tetanic stimulation of the perforant path with a concomitant decrease in the number of non-concave spine heads (Desmond and Levy, 1983; 1986; 1988). Chang and Greenough (1984) proposed that the maintenance of LTP was associated with a transition from shaft to spine synapses. Presynaptically, a redistribution of synaptic vesicles with a significant increase in attached vesicles and vesicles adjacent to the active zone, have been demonstrated in Schaffer-commissural inputs following the induction of LTP (Applegate et al., 1987). This is likely to be associated with an increase in the number of synaptic vesicles per terminal following the induction of LTP (Meshkul and Hopkins, 1990).

The use of protein synthesis inhibitors has demonstrated that protein synthesis is involved in the maintenance of the later phase of LTP (Matthies,

1989). In fact, an increase in incorporation of labelled valine into area CA1 and the dentate gyrus after induction of LTP have been shown (Duffy et al., 1981). In addition, there was an increase in released proteins following the induction of LTP in the dentate gyrus (Duffy et al.,1981; Fazeli et al., 1990). Moreover, the release of proteins of molecular weight 14-64 KDa which occured 2-4 hours after tetanus, was blocked by perfusion with APV (Fazeli et al., 1990). However, in anisomycin-treated mice, LTP-associated morphological changes are inhibited 4 minutes, but not 90 minutes, after tetanic stimulation of the perforant path (Fifková et al., 1982) suggesting that an early and critical time period of protein synthesis in the maintenace of LTP is necessary. LTP and immediate-early gene activation has been shown in some cases (see later).

Glycoproteins have been shown to be important in the maintenance of LTP. If 2-deoxy-D-galactose (2-Dgal), which inhibits glycoprotein synthesis, is administered either before or after tetanisation, *in vivo* or *in vitro*, then LTP is induced as normal but it rapidly decays (Krug et al., 1991). Some of these glycoproteins could be neural cell adhesion molecule (NCAMs) which are considered to be important in synapse strengthening.

One of the major conceptual problems of LTP as a model for memory is that it can last so long (up to days). Whereas it is clear from hippocampal lesion studies in the rat that this structure is important in the acquisition and retention of a memory (Peinado-Manzano, 1990). A solution to this could be in the discovery of a phenomena called long-term depression (LTD). LTD like LTP serves to regulate synaptic strength via a prolonged inhibition of synaptic transmission. It is thought that LTD functions to "switch-off" LTP. However, it is not the purpose of this thesis to enter into the debate as to whether LTP is a real phenomenon or not. In its simple form as a model for synaptic plasticity, it has been shown to induce immediate-early genes (Cole et al., 1989; Wisden et al., 1990; Richardson et al., 1992; see later).

AVIAN LEARNING

Although birds have occasionally been elevated to honorary mammalian status (Thompson et al., 1983), the relevance of avian learning and memory to this thesis warrants separate treatment.

Song-learning

Song learning in canaries and finches also requires memory formation, in this case, auditory feedback is also required for complete acquisition of song behaviour (Nottebohm, 1977). Song is produced by the syrinx, a structure in the neck that is innervated by the left and right hypoglossus nerves. Section of the left hypoglossus nerve leads to deficits in vocalisation, whilst section of the right hypoglossus nerve does not have such a profound effect on song (Nottebohm, 1972; 1984). If, however, the left hypoglossus nerve is cut before spring (i.e. before the song-generation season), the right hemisphere will assume dominance for song control. Further lesion studies identified the importance of the neostriatum and rostral hyperstriatum ventrale (HVc, later named the higher vocal centre) in song behaviour (Nottenbohm et al., 1976).

Large sex differences in the morphology of the song-bird brain have been identified. Female canaries have a less well-developed area X compared to males, and in the female zebra finch area X is non-existent while apparent in males. In canaries the size of the nucleus in both males and females was found to correlate with singing behaviour (Nottebohm and Arnold, 1986). The sex differences in brain structure appear to be under the control of hormones; the growth of HVc and RA could be induced in females by the injection of testosterone (Nottebohm, 1980). The amount of testosterone in males undergoes seasonal changes which are correlated with changes in the size of the testes, the relative volumes of RA and HVc and with the size of song repertoire (Nottebohm et al., 1981).

The fluctuation in the size of song-related nuclei were interpreted as reflecting changes in the number of synapses in these regions; a greater number would facilitate connectivity between neurones, the formation of new neuronal networks and the representation of a more complex song repertoire (Nottebohm, 1984). Indeed, the volume of song nuclei was found to increase in the successive development from sub-song, through plastic song to full or stable song (Nottebohm et al., 1986).

Golgi studies of the hormone-sensitive type IV neurones of the RA region of female canaries forebrain revealed that hormones that induced song development also results in the addition of new synapses throughout the dendritic tree (Canady et al., 1988). This suggests that increased connectivity requirements may indeed underlie the increased volume of the nucleus.

The results of studies examining morphological differences in the brain of male and female song birds pose interesting questions concerning the representation of information in the brain. Clearly the male does not have larger song nuclei to represent more information since the female must have equally complex representations in order to recognise and respond to the song. It appears that the morphological sex differences reflect the way in which stored information is utilised.

Although studies of the neurobiology of song-learning have revealed much about the neural substrates of the behaviour, the extent to which song acquisition and recognition can be regarded as learning is unclear.

Food-storing

Food storing and retrieval behaviour in birds has been studied. Some species store their food at one or two sites whereas others store in multiple sites. In order to recall the location of their "stash", birds must be laying down

some sort of memory trace. Post-storing lesioning of the hippocampus (HP) causes a deficit in the bird's ability to find its stash (Clayton and Krebs, 1994). There is also a correlation between relative hippocampal size and storing ability (Healy et al., 1991).

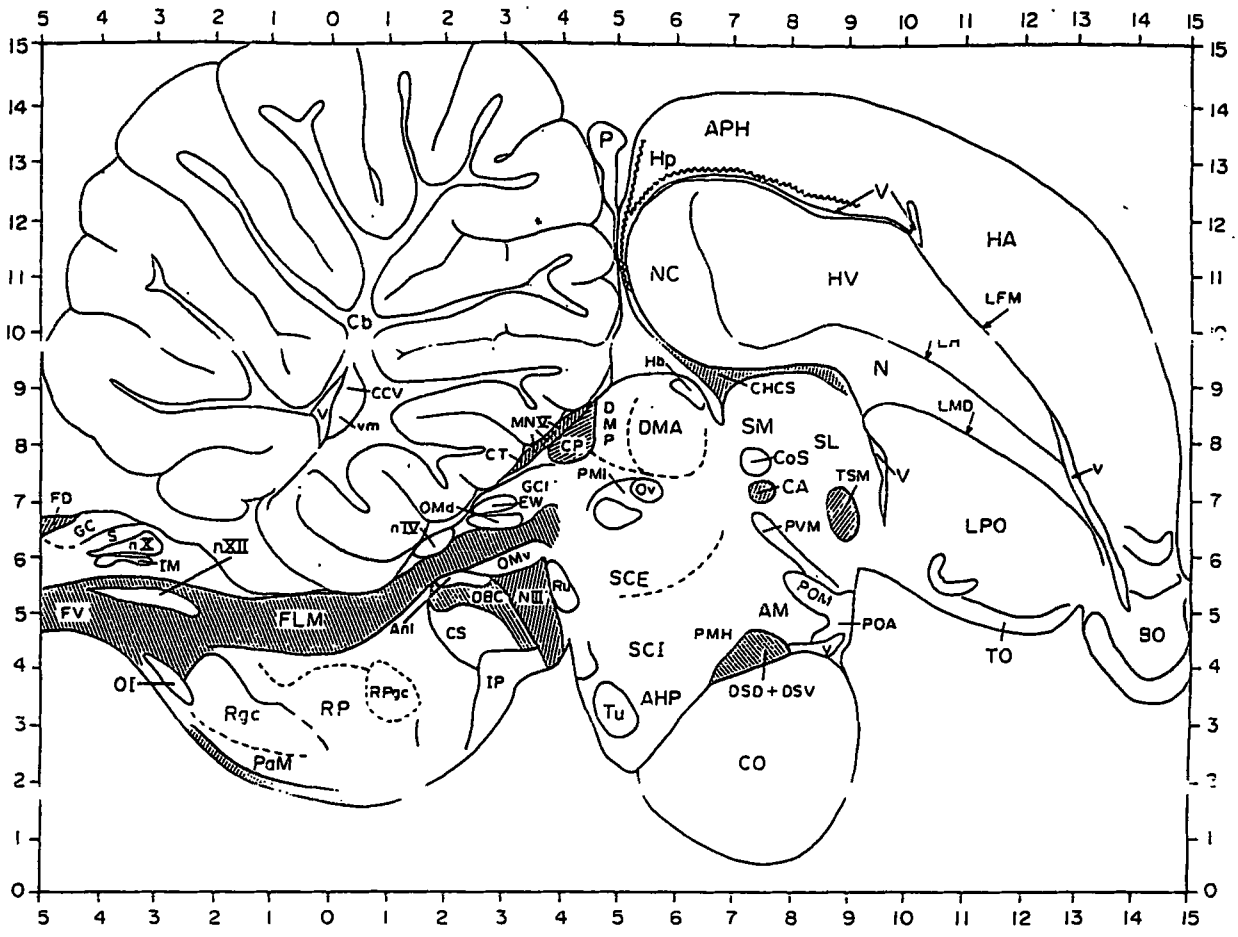
Mapping of active areas by anti-Fos immunocytochemistry in marsh tit after food storing has shown that the intermediate medial hyperstriatum ventrale (IMHV), archistriatum (Arch) and lobus parlofactorius (LPO) are active (Szekely et al., 1992).

The Chick Model

Learning and memory formation in the domestic chick (*Gallus gallus domesticus*) has been studied extensively. These are attractive animals to study because they are inexpensive, small and so can be housed in large numbers. They are also precocious; capable of independent exploration and learning within 12 hours of hatching. In addition, their blood-brain barrier is minimal and their skulls are unossified, allowing the easy administration of pharmacological agents via either intracranial or systemic routes. Another advantage of the young chick is that the "signal" of the memory trace is less likely to be lost in the "noise" of what the animal has already learnt about its environment.

Neuroanatomy of the chick brain

Different regions of the chick brain have been identified (fig. 1.1.). Several of these regions are homologous (morphology)/analogous (function) to regions in the mammalian brain.



AHP	Area hypothalami posterioris
AM	Nucleus anterior medialis hypothalami
Anl	Nucleus annularis
APH	Area parahippocampalis
BO	Bulbus olfactorius
CA	Commissura anterior
Cb	Cerebellum
CCV	Commissura cerebellaris ventralis
CHCS	Tractus cortico-habenularis et cortico-septalis
CO	Chiasma opticum
CoS	Nucleus commissuralis septi
CP	Commissura posterior
CS	Nucleus centralis superior (Bechterew)
CT	Commissura tectalis
DBC	Decussatio brachiorum conjunctivorum
DMA	Nucleus dorsomedialis anterior thalami
DMP	Nucleus dorsomedialis posterior thalami
DSD	Decussatio supraoptica dorsalis
DSV	Decussatio supraoptica ventralis
EW	Nucleus of Edinger-Westphal
FD	Funiculus dorsalis
FLM	Fasciculus longitudinalis medialis
FV	Funiculus ventralis
GC	Nuclei gracilis et cuneatus
GCt	Substantia grisea centralis
HA	Hyperstriatum accessorium
Hb	Nucleus habenularis
Hp	Hippocampus
HV	Hyperstriatum ventrale
IM	Nucleus intermedius
IP	Nucleus interpeduncularis
LFM	Lamina frontalis suprema
LH	Lamina hyperstriatica
LMD	Lamina medullaris dorsalis

LPO	Lobus parolfactorius
MNV	Nucleus mesencephali nervi trigemini
N	Neostriatum
NC	Neostriatum caudale
NIII	Nervus oculomotorius
nIV	Nucleus nervi trochlearis
nX	Nucleus motorius nervi vagi
nXII	Nucleus nervi hypoglossi
OI	Nucleus olivaris inferior
OMd	Nucleus nervi oculomotorii, pars dorsalis
OMv	Nucleus nervi oculomotorii, pars ventralis
Ov	Nucleus ovoidalis
P	Corpus pineale
PaM	Nucleus paramedianus
PMH	Nucleus medialis hypothalami posterioris
PMI	Nucleus paramedianus internus thalami
POA	Nucleus preopticus anterior
POM	Nucleus preopticus medialis (van Tienhoven)
PVM	Nucleus periventricularis magnocellularis
Rgc	Nucleus reticularis gigantocellularis
RP	Nucleus reticularis pontis caudalis
RPgc	Nucleus reticularis pontis caudalis, pars gigantocellularis
Ru	Nucleus ruber
S	Nucleus solitarius
SCE	Stratum cellulare externum
SCI	Stratum cellulare internum
SL	Nucleus septalis lateralis
SM	Nucleus septalis medialis
TO	Tuberculum olfactorium
TSM	Tractus septomesencephalicus
TU	Nucleus tuberis
V	Ventriculus
vm	Nucleus cerebellaris internus, pars ventromedialis

Figure 1.1. Schematic diagram of chick brain anatomy (Youngren & Phillips, 1978).

A major problem with attempting to identify neuronal pathways in the immature chick's forebrain is that it is very plastic, with some connections that exist in the young chick disappearing in the adult brain. For example, an asymmetry of the thalamic projections, which exists in the young chick forebrain, is only transient and disappears after day 20 post-hatching (Boxer and Stanford, 1985; Rogers and Sink, 1988). Hence, when identifying neuronal pathways, this plasticity must be take this into account.

Morphologists have attempted to identify pathways that connect the intermediate hyperstriatum ventrale (IMHV) and lobus parolfactorius (LPO); regions implicated in avian memory formation (Rose, 1993). This is complicated by the fact that as yet no monosynaptic pathway between the IMHV and LPO has been identified. Hence the existence of polysynaptic pathways between these two structures have been sought, involving other forebrain structures. Histologically, the avian telencephalon is a laminated structure. The dorsal laminae are collectively known as the hyperstriatum and comprise the hyperstriatum acessorium, intercalatum, dorsal, and ventral. The dorsal regions of the hyperstriatum form the visual Wulst. The Wulst, together with the ectostriatum, are major primary telencephalic visual projection areas. The archistriatum (ARCH) contributes to descending motor pathways as well as to the avian limbic system (Zeier and Karten, 1971). Parts of the neostriatum receive inputs from auditory (Karten, 1968), somatic sensory (Delius and Bennetto, 1972), and visual (Parker and Delius, 1972) pathways. The IMHV receives inputs from most sensory modalities and projects to brain regions involved in motor control and in aspects of antagonistic behaviour (Bradley et al., 1985). Davies (1991) has argued that the IMHV's role in memory formation is primarily concerned with object recognition. The IMHV has reciprocal projects to the visual Wulst, ARCH and paleostriatum augmentatum (PA; Bradley et al., 1981; Davies et al., 1991). In addition it receives afferents from the hippocampus (HP), area septalis, neostriatum, and

the caudal part of the hyperstriatum ventale. The IMHV sends efferents to the posterior part of the ARCH and to the dorsolateral part of the cerebral hemispheres. A number of these connections are bilateral. Thus the IMHV is ideally placed to act as an integrative centre in the telencephalon, linking sensory and motor systems and is subject to influences from regions of the brain thought to be concerned with motivational aspects of behaviour.

In learning and memory the two main disynaptic routes that are considered to link IMHV and LPO are the efferent pathways to the ARCH and PA. Here the ARCH and/or PA could in some way mediate the transfer of newly acquired information from the IMHV to long-term storage in the LPO (Davies et al., 1991). There is evidence that the IMHV plays a role in visual discrimination tasks relating to food selection (Andrew, 1991). If a chick pecks at a bitter tasting bead it will evince a disgust response and avoid subsequent presentations of a similar but dry bead (one-trial passive avoidance training). In this learning task the IMHV could process information about the appearance and shape of a training bead, and its unpleasant nature, in an association-like role. Since the ARCH is involved with processing information regarding fear responses (Phillips and Youngren, 1986), it seems likely that this area is also involved in the learning of a passive avoidance tasks. The LPO, together with the PA, forms part of the avian paleostriatal complex which is believed to be homologous with the mammalian basal ganglia, the caudate-putamen and globus pallidus; structures involved in modification of efferent output (Dubbeldam, 1991). Individual neurones in the IMHV have been reported to project to both ARCH and PA (Bradley et al., 1985). Hence, both areas maybe involved with passive avoidance learning, with the PA mediating the motor aspects of performing the task and the ARCH having a "motivational" role which could affect the chick's ability to learn (Lowndes and Davies, 1994). The LPO could be involved in inhibition of natural pecking behaviour on presentation of the adversive stimulus (eg. chrome bead). It also

appears that the IMHV may retain a role in colour discrimination but the process of recognition of the bead as a "bad object" is most likely to be a function held within other, yet undefined, forebrain structure (Stewart and Rusakov, 1994). Figure 1.2. shows the main known pathways that are thought to be important in learning and memory.

Figure 1.2.

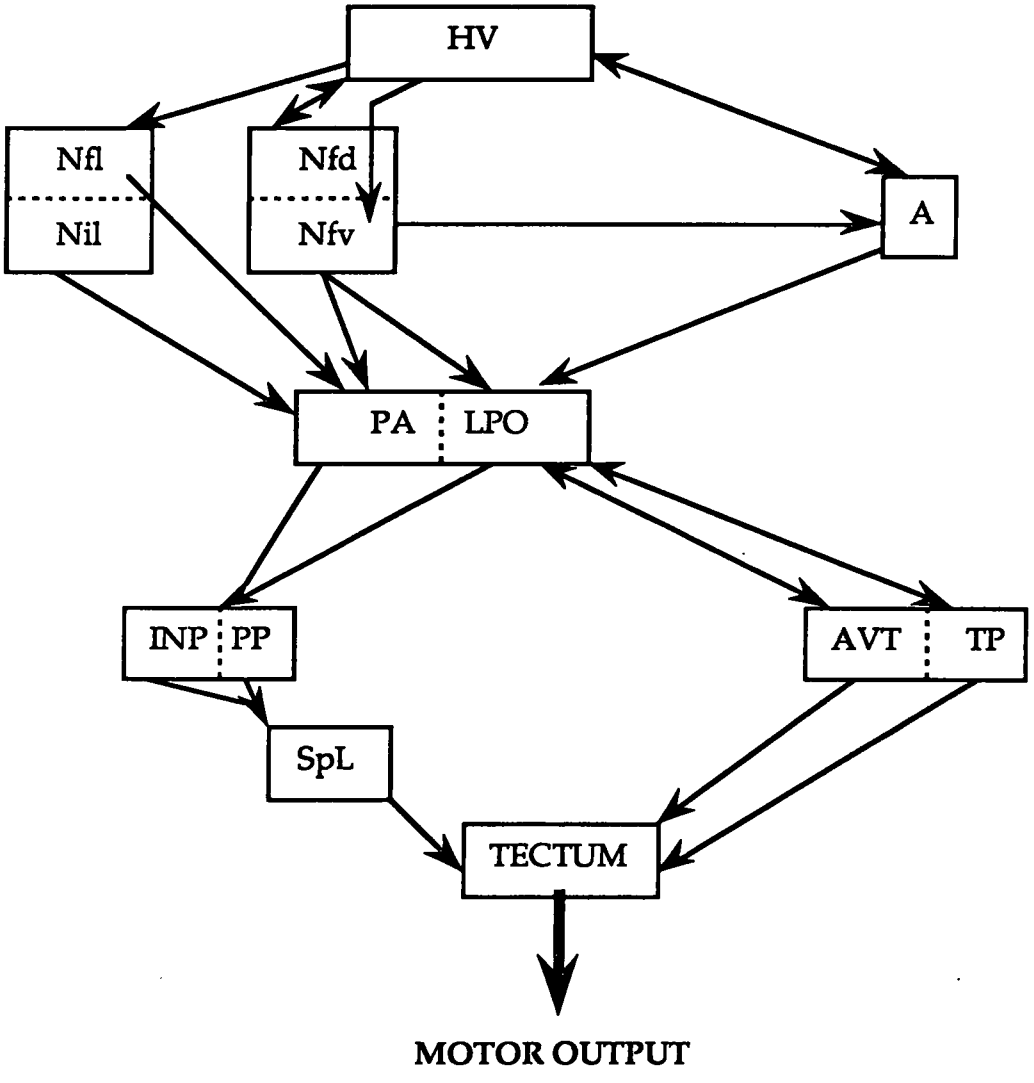


Diagram to show some of the principal connections between the hyperstriatum ventrale (HV) and the LPO in relation to tectal motor output. For simplicity, the reciprocal connection between the HV and palaeostriatum accessorium (PA) is not shown. Through their connectivity, the archistriatum

and the neostriatum are two regions that have the potential to control and possibly synchronize the activities of the IMHV and LPO. A, archistriatum; Nfl, Nfd and Nfv, lateral, dorsal and ventrale aspects of the neostriatum frontale; Nil, lateral aspect of the neostriatum pars intermedia; INP, nucleus intrapeduncularis; PP, paleostriatum primitivum; AVT, area ventralis; TP, nucleus tegmenti pedunculo-pontinus; SpL, nucleus spiriformis (diagram courtesy of J. Gigg et al., 1994).

IMPRINTING

Many young birds are particularly good at learning the visual and auditory characteristics of the mother during the first few post-hatch days. This is called imprinting and lasts indefinitely. Imprinting is an important natural behaviour adopted by the newly hatched chick because it affords it with its mother's protection. Morphological and biochemical changes have been found to be associated with both visual and auditory imprinting (Horn, 1985; Scheich, 1987; McCabe and Horn, 1988).

The imprinting paradigm employed by Horn's Cambridge group is briefly as follows. Dark reared chicks are exposed for a period of time to either a flashing light, a rotating box or a stuffed jungle fowl. Chicks can be tested for "recall" of the imprinting stimulus at different times after exposure. The degree of imprinting is scored as the chick's preference for the original object that it was exposed to compared to another object presented at the same time as testing.

After imprinting, an increase in the incorporation of uracil (Bateson et al, 1972) and lysine (Horn et al., 1973) has been demonstrated in the day-old chick. Autoradiographic studies indicated that uracil incorporation and 2-deoxyglucose (2-DG) uptake were greatest in the IMHV of trained chicks

(Kohsaka et al., 1979). C-*fos* mRNA has been shown to be expressed in the IMHV, hippocampus, medial neostriatum, paleostriatum, stratum opticum and cerebellum after imprinting (Abramova et al, 1992).

Morphologically, imprinting causes an increase in the length of the post-synaptic density (PSDs) in spine synapses of the left IMHV (Horn et al., 1985). In addition, there is an increase in the number of NMDA receptors in the left IMHV but not the right IMHV (McCabe and Horn, 1988). Spontaneous electrical activity in the IMHV, which may reflect synaptic modification, decreases with training but increases in the hyperstriatum accessorium after training on the red box only (Payne and Horn, 1982).

Lesion studies to investigate the role of the IMHV were conducted. Removal of the entire lateral forebrain prevented acquisition and retention of an imprinted preference (Saltzen et al., 1975; 1978). This deficit was later localised to the IMHV (McCabe et al., 1981). Pre-training lesions prevented the acquisition of the imprinting task, whereas post-imprinting lesions had a less pronounced effect, suggesting that the IMHV had, in addition, a role in retention (McCabe et al., 1982). In fact, bilateral lesions of the IMHV up to 6 hours post-training (i.e. exposure to an imprinting stimulus) caused the chicks to be amnesic, whereas lesioning after this time resulted in recall (Davey et al., 1987; Davey, 1988).

Cipolla-Neto et al (1982) found that if they lesioned the right IMHV at various time-points up to 3 hours after training, the chicks showed retention. If the same chick then had its left IMHV lesioned 26 hours after training, it was rendered amnesic. This result suggested that the right IMHV plays a role in acquisition and the left IMHV in retention of the imprinting task. However, when the left IMHV was lesioned less than 3 hours after training, there was no amnesia; and if the remaining right IMHV was lesioned after the left, the chick

continued to show retention. These results suggested that the right IMHV played a role in acquisition of the task and that both left and right IMHV participate in retention.

Since the morphological and receptor binding effects were restricted to the left IMHV, the left IMHV was proposed to be a storage site of information about the training stimulus (McCabe, 1991). It was also suggested that the right IMHV acts as a different kind of store than that of the left IMHV, perhaps a temporary or buffer store. It was further suggested that another storage depot existed in the right hemisphere termed S', during the 26 hours after training (Cipolla-Neto et al., 1982). Thus, the right IMHV would be required for acquisition and the left IMHV critical for storage, with S' being able to support retention in the absence of the left IMHV. Further lesion studies showed that the integrity of the right IMHV, and not the abscence of the left IMHV, was necessary for storage in S'.

Pretraining unilateral abalations of the right IMHV did not impair imprinting, whereas subsequent abalation of the left IMHV resulted in amnesia. If the left IMHV was lesioned prior to training, imprinting was not impaired. However, subsequent lesioning of the right IMHV did result in amnesia, suggesting that no storage in S' had occurred. A possible explanation for this unexpected result could be that in the pretraining absence of the left IMHV, the right IMHV "takes-over" the role of the left IMHV. It is also possible that the right IMHV is incapable of performing both functions (S') simultaneously; the mechanism for storage in S' is "lost" (McCabe, 1991).

Pharmacological studies also suggested that cellular mechanisms subserving imprinting to different classes of objects, are different. Injections of the toxin DSP4, which reduces noradrenalin concentrations in the brain, resulted in impaired imprinting to a rotating box but not to a stuffed jungle fowl (Davies

et al., 1985). Therefore noradrenergic innervation might play a greater role in imprinting on a box than an animal-like figure. An increase in cholinergic receptor binding has also been demonstrated in trained chicks (Bradley and Horn, 1981). Aminergic systems in the brain are also known to affect motivation (Mason, 1984). The behavioural effect of the toxin might, therefore, stem from reduced motivation to imprint on an unnatural stimulus, rather than from defective learning. Conversely, injections of testosterone enhanced imprinting on the fowl but not on the box, indicating that different mechanisms were being used for encoding the different visual stimuli (Bolhuis et al., 1986).

PASSIVE AVOIDANCE TRAINING

One-trial passive avoidance training is an attractive model because one can time the exact point that the animal experiences the training task and hence commencement of the memory formation cascade. This task exploits the precocity of newly-hatched chicks who explore their environment by pecking at small attractive objects. The chick rapidly learns to distinguish between food and non-food items such as faeces. Chicks are trained according to the one-trial passive avoidance paradigm previously described by Lössner and Rose (1983). Briefly, chicks are pretrained on a small white bead (2.5 mm diameter) in order to determine whether their pecking behaviour is normal. If the chick pecks at least twice out of three separate pretraining trials then it is trained on a slightly larger chrome bead (4 mm diameter) dipped in the bitter tasting substance, Methylanthranilate (MeA). The chick pecks at the bead and displays a typical disgust response of wiping its beak on the floor and shaking its head. At any time after training the chick is tested for recall (avoidance of the bead) or amnesia (pecking of bead) by presentation of a similar but dry chrome bead. A control for this paradigm involves the training of chicks on a bead dipped in Water rather than MeA.

Researchers at the Brain Research Group (and other groups) have identified many consequences of one-trial passive avoidance training in the chick. The findings detailed below refer to significant effects found in birds trained on the MeA bead compared to control birds who are presented with a Water-coated bead and continue to peck at the dry bead on test.

Stages of Memory Formation

Memory in the chick has been claimed to consist of 3 sequential stages (Gibbs et al., 1973; 1978). Short-term memory (STM), lasting for the first 10 minutes after training. Administration of large doses of the inhibitor glutamate, which disrupts membrane hyperpolarization, during this sensitive period results in amnesia for the task. STM is considered to reflect an immediate post-tetanic hyperpolarisation caused by potassium. Intermediate-term memory (ITM), lasting from 10-30 minutes post-training, is thought to involve hyperpolarisation due to the sodium/potassium pump. Injections of ouabain, which blocks this pump, are effect at causing amnesia up to 30 minutes post-training. Finally, long-term memory (LTM) formation, commencing from 30 minutes onwards of training, is believed to involve *de novo* protein synthesis since injection of the antibiotic cycloheximide around training does not produce amnesia until approximately 1 hour after training (Gibbs and Barnet, 1976). These stages are considered to be sequential because inhibition of the early stages also inhibits the later stages of memory formation. However, inhibition of the later stages does not inhibit the preceding stages (Gibbs and Ng, 1977; Patterson et al., 1986).

Additional evidence for the three sequential stages of memory formation is described below. Populations of chicks trained on the passive avoidance paradigm display two time-points at which retention is temporarily reduced. These dips occur at 15 minutes and 55 minutes post-training and are

considered to reflect the interface from STM to ITM and ITM to LTM respectively (Gibbs and Ng, 1979). Patterson et al (1986; 1988) found similar time-windows for STM and ITM using glutamate and oubain, respectively. However, they used a different inhibitor of protein translation, anisomycin, and found that the onset of LTM occurred later than 55 minutes (Gibbs and Ng, 1979) at between 60-90 minutes post-training, with full amnesia only developing after 75 minutes. Differences in housing regimes have been shown to extend the boundary between ITM and LTM up to 70 minutes (Gibbs and Ng, 1979).

ITM has been found to consist of at least two phases, an energy-dependent phase A and an energy-independent phase B. ITM lasts from 15 to 55 minutes after training and intracranial injection of the metabolic inhibitor 2,4,-dinitrophenol (DNP) at any time up to 25 minutes after training disrupts memory formation for the task. However, if the injection is given 30 minutes or more after training then DNP has no effect. Therefore, phase A can be abolished by DNP and lasts about 10 minutes; phase B is not affected by DNP and lasts approximately 25 minutes (Gibbs and Ng, 1984b). It is thought that the neuronal processes underlying the development of LTM, which are susceptible to inhibition by antibiotics, are initiated at the time of transition from phase A to phase B of ITM. If chicks are trained on a weakly-reinforced version of the passive avoidance task, obtained by diluting MeA in alcohol, one sees evidence of STM and ITM (A), but not of ITM (B) or LTM (Ng and Gibbs, 1991). Hormones may also play a role in determining the transition time from phase A to phase B (Gibbs et al., 1991). In fact, the duration of each memory phase is thought to be under hormonal control because corticosterone, adrenocorticotrophic hormone$_{1\text{-}24}$, and testosterone lengthen the duration of STM and ITM and extend the onset of the LTM stage (Gibbs and Ng, 1984; Gibbs et al., 1986). Patterson et al (1986) have found similar results.

Location of Activity

2-deoxyglucose (2-DG) is an analogue of glucose and, like glucose, is taken up by metabolising cells. However, it cannot be fully metabolised via the glycolytic pathway and so accumulates in metabolically active cells. If 2-DG is radiolabelled, one can located the brain regions in which it accumulates, the degree of radioactivity for each area being proportional to metabolic activity. Injection of 2-DG prior to MeA training results in an increase in accumulation in the IMHV, paleostriatum augmentatum (PA) and LPO 30 minutes post-training (Kossut and Rose, 1984). Uptake in the left IMHV was found to be greater than that in the right IMHV. Injections of 2-DG 10 minutes post-training led to increased accumulation in the LPO, but not when injected 30 minutes post-training (Rose and Csillag, 1985). This increased accumulation is believed to represent greater activity in these brain regions in response to passive avoidance learning.

Receptor activation

There is enhanced uptake of 45-calcium into prisms prepared from the IMHV within the first 30 minutes after training. Bilateral injection of the N-type calcium channel blocker, ω-conotoxin, directly into the IMHV has been shown to cause amnesia *in vivo* and inhibit the training-related influx of calcium in the left (but not right) IMHV *in vitro* (Clements and Rose, 1994). A spatial re-arrangement of vesicles at 30 minutes post-training has been found in the synapses of both left and right IMHVs (Rusakov et al., 1993). In addition there is enhanced release of glutamate in the left IMHV (Daisley and Rose, 1993). This could represent the release of endogenous amino acid from the vesicular transmitter pool (Nicholls and Sihra, 1986), derived from an increased recruitment of the neurotransmitter-containing vesicles. Also at this time point there is a transient upregulation of NMDA receptor activity (Stewart et al., 1992). Injection of MK-801, a non-competitive antagonist of

NMDA receptor function, has been shown to be amnestic, i.e. upon test, the chick shows no recall for the training experience by pecking at the bead (Burchuladze and Rose, 1992). Similarly, if the binding of glycine to its regulatory site on the NMDA receptor is inhibited by 7-chlorokynurenine (7-ClK) the chicks are also rendered amnesic for the task. Bilateral intracranial injections of 7-ClK have shown that only the left hemisphere is crucial for retention of this task (Steele and Stewart, 1993). By contrast, no amnesia resulted if non-NMDA glutamate receptors were blocked by antagonists such as CNQX (Burchuladze and Rose, 1992). Using various glutamate receptor antagonists and measuring inositol phosphate turn-over, Bullock et al (1993) have shown that there is a greater number of NMDA receptors in the left IMHV than the right IMHV.

Nitric oxide (NO) is involved in the early stages of memory formation since pre-training injections of inhibitors of NO such as nitroarginine result in amnesia for the task within 30 minutes of the training trial. It is thought that NO may be functioning as a retrograde secondary messenger, being released postsynaptically, facilitating further release presynaptically (Hölscher and Rose, 1992; 1993). However, further work needs to be carried out to determine the exact role that NO plays in memory formation.

Protein Kinases

The role of protein kinases, especially PKC, in memory formation has been extensively studied. PKC has a ubiquitous role in the cell, being involved in phosphorylation of many proteins that are involved in the memory cascade. Within the first 30 minutes after training the degree of phosphorylation of the presynaptic PKC substrate B50 is increased in right upper forebrain samples (this includes the IMHV). It is also thought that PKC is translocated from cytosol to membrane in response to training (Ali et al., 1988a; 1988b;

Burchuladze et al., 1990). Injection of inhibitors of PKC such as mellitin around the time of training cause amnesia for the task. But this amnesia does not manifest itself until 3 hours post-training, that is, well after the significant changes in phosphorylation (30 minutes post-training). This suggests that PKC-phosphorylation of the B50 protein is involved in the processes that make the transition from STM to LTM possible (Burchuladze et al., 1990).

The role of PKC in later events could involve the phosphorylation of molecules required for IEG activation, transcription factors and late gene products. It has been shown that Fos undergoes extensive post-translational modification, mainly phosphorylation of serine residues (Morgan and Curran, 1991).

Nucleus

It is thought that the consequence of NMDA receptor activation is an increase in the concentration of intracellular calcium. This in turn can directly or indirectly stimulate immediate-early gene (IEG) activation. In fact, Anokhin and Rose (1990) demonstrated that c-*fos* mRNA was induced 30 minutes after training in both left and right IMHV and LPOs. In a second experiment using the appetitive learning paradigm of the pebble floor, chicks were given the opportunity to peck for 10 minutes at scattered non-food items (very small pebbles stuck to the floor of the training pen) and food items (chopped, boiled egg white). The number of pecks at food and non-food items were recorded over this time period. It became evident that the chick rapidly learns to discriminate between these food and non-food items, preferentially pecking at egg white. Learning to discriminate between pebbles and egg white causes an induction of both c-*fos* and c-*jun* mRNA in chick forebrain (Anokhin et al., 1991). The consequence of IEG activation is activation (or repression) and

expression of late genes that give rise to the biochemical and morphological changes seen further down-stream of training.

Glycoproteins

Fucose is one of the major sugar units found in glycoproteins. Intracerebral injections of radiolabelled fucose showed an increased incorporation into glycoproteins of the synaptic plasma membrane (SPM), in forebrain roof (the area containing the IMHV) and right forebrain base (the area containing the LPO) of birds MeA-trained. This persisted for at least 24 hours following training (Sukumar et al., 1980; Burgoyne and Rose, 1980). In addition, fucokinase levels were also elevated (McCabe and Rose, 1985). This fucose incorporation appears to be a post-translational event because the protein synthesis inhibitor cycloheximide was ineffective at inhibiting fucose incorporation. Administration of a short sub-convulsive transcranial electroshock to the chick will temporarily alter membrane potentials thereby interfering with normal synaptic transmission. If this shock is applied just after the training task then the chick is amnesic for the training experience 30 minutes post-training. However, if the shock is delayed till 10 minutes post-training then the birds exhibit recall upon test 30 minutes after training. In chicks that were electroshocked just after training, and who also had recall, there was an increased fucose uptake in areas previously mentioned (forebrain base and anterior forebrain roof) whereas no increase in fucose incorporation was observed in birds that failed to recall the experience (Rose and Harding, 1984). Sub-convulsive shock *per se* does not affect the rate of fucose incorporation into macromolecules between 45 minutes and 3.75 hours after either Water- or MeA- training. It was therefore considered that increased fucose incorporation was not due to a non-memory related consequence of the training procedure eg. stress or increased motor activity.

Fucose incorporation into glycoproteins is prevented by the anti-metabolite 2-deoxygalactose (2-Dgal). Intracranial injections of 2-Dgal, when administered between 2 hours pre- and 2 hours post- training causes amnesia for the task 24 hours later (Bullock et al., 1990). Glycoproteins whose synthesis is inhibited by 2-Dgal are thought to include neural-cell adhesion molecules (N-CAM). In the hours following training the molecular cascade continues with the synthesis of a number of pre- (50 kDa) and post- (100-120 and 150-180 kDa) synaptic membrane glycoproteins (learning-associated glycoproteins; LAGs). These glycoproteins are believed to include N-CAMs (Scholey et al., 1993). N-CAMs are considered to be important in the synaptic remodelling underlying memory. Injection of antibodies directed against N-CAMs at 5.5 to 8 hours post-training or its putative associate, the cell adhesion molecule, L1 at both 30 minutes pre- and 5.5 to 8 hours post- training, prevents the chick from having recall of the training experience 24 hours later (Scholey et al., 1993; 1994).

Waves of Neuronal Activity

Two waves of neuronal activity have been observed after passive avoidance training; the first lasting up to 90 minutes after training and the second between 4 - 8 hours post-training. Both waves include sensitivity to 2-Dgal (Bullock et al., 1990; Scholey et al 1992). The spontaneous neuronal bursting observed in many areas of the chick forebrain is also significantly increased in both IMHV and LPO after training. This too appears to follow a biphasic pattern (Gigg et al., 1994). The first wave is considered to involve the modification of existing proteins. Crowe et al (1994) showed that pretraining injections of 2-Dgal caused amnesia for the task 40 minutes after training, whereas pre-training injections of anisomycin only started to cause amnesia 60 minutes post-training. Hence, the time of onset of sensitivity to 2-Dgal (glycoprotein synthesis) precedes that of anisomycin (protein synthesis). Injection of an antibody directed against the cell adhesion molecule L1 at

different times relative to training caused a similar biphasic response as that seen due to 2-Dgal (Scholey et al., 1994). However, by using polyclonal antibodies that recognise N-CAMs, Scholey et al (1993) have found that intracranial injections of this antibody are only amnestic during the second wave of activity, at 5.5-8 hours after training. It is hypothesized that the second wave of glycoprotein synthesis only occurs if the training stimulus is strong/adverse enough. This has been demonstrated with chicks trained on the passive avoidance task using the less adversive stimulus, quinine. Training with quinine showed no increase in fucose uptake during this second period of 2-Dgal sensitivity (Bourne et al., 1991).

Neurophysiologically an increase in high-frequency neuronal bursting has been observed in the IMHV of anaesthetised chicks at both 3-4 and 6-7 hours post-testing (n.b. testing occurs 1 hour post-training). This enhanced neuronal bursting at 3-4 hours post test embraces both left and right IMHV. Whereas the second period of enhanced bursting (6-7 hours post-test) is confined to the right IMHV (Gigg et al., 1993). An increase in electrical bursting is also seen in both left and right LPO 4-7 hours post-test (Gigg et al., 1994). It has been suggested that these two periods of neuronal activity are due to an initial activation of the left IMHV followed by a redistribution to the right IMHV and LPO (Patterson et al., 1990).

Lesion Studies

The effect of lesioning areas of the chick brain on its ability to recall the training experience have been studied. Pre-training bilateral lesions of the IMHV resulted in amnesia for the training task, whereas post-training lesions did not affect the chick's ability to recall. This led to the conclusion that the IMHV is necessary for acquisition, but not for storage and retention, of the passive avoidance memory. Pre-training unilateral IMHV lesions showed that

the left IMHV (and not the right IMHV) was required for memory acquisition (Patterson et al., 1990). Furthermore, pretraining lesions of the left hippocampus decreased the chick's ability to recall whilst lesioning 1 hour after training does not affect recall. This suggests that the left hippocampus is also required for acquisition of the training task (Sandi et al., 1992). In certain respects this is not surprising considering that the IMHV recieves monosynaptic afferents from the hippocampus (Bradley et al., 1985).

Bilateral pre-training LPO lesions did not result in amnesia for the task. However, bilateral LPO lesions 1 hour after training did render the chick amnesic when tested 24 hours after training. Unilateral lesions did not affect retention for passive avoidance learning, suggesting that any part of the LPO was sufficient for recall (Gilbert et al., 1991). The ARCH recieves efferents from the IMHV and also projects to the the LPO (Bradley et al., 1985). Hence, the ARCH may be acting as a "relay-station" between IMHV and LPO. The ARCH has been implicated with fear and avoidance behaviour (Phillips and Youngren, 1986). Pre-training bilateral lesions of the ARCH prevented recall for the passive avoidance training task, suggesting that the ARCH is involved in acquisition of this task (Lowndes and Davies, 1994). Whether acquisition is lateralized is yet to be determined since unilateral lesioning of the ARCH have yet to be performed. Lesioning results, however, must be treated with some caution because one cannot automatically equate lack of function with absence of one particular brain region.

Morphology

Electron microscopy has revealed that 24 hours after training there is an increase in the presynaptic bouton density in the left IMHV compared to the right IMHV in MeA- but not Water- trained birds. In Water trained birds there were more vesicles per unit volume of neuropil in the right compared to the

left IMHV; the opposite was the case for MeA birds. The mean length of postsynaptic thickening was greater in the right IMHV compared to the left in Water chicks and this asymmetry was abolished in MeA trained birds. The most dramatic result reported in MeA chicks was that the left IMHV contained more vesicles per synapse than the right IMHV (Stewart et al. 1984).

Similar studies were undertaken in the LPO. An increase in the numerical density of synapses was observed in both left and right LPO of Water trained chicks, compared to MeA trained chicks. The synaptic thickening was greater in the right compared to the left LPO of Water chicks while the reverse was found for MeA birds. No differences were found in the volume density of presynaptic boutons or in the mean bouton density when between-hemispheres and between-training group comparisons were made. Both the numerical density of synaptic vesicles and the number of vesicles per bouton were greater in the left LPO of MeA compared to Water chicks. No differences were found in measures of mean synaptic curvature, mean length of synaptic thickening, numeric densities of synaptic synapses or the volume density of presynaptic boutons. The mean bouton volume was greater in the left LPO of MeA chicks compared to Water trained controls. The numerical density of synaptic vesicles, and the number of vesicles, per presynaptic bouton was greater in the right LPO compared to left in Water trained birds; training on the MeA bead appeared to abolish this asymmetry (Stewart et al., 1987).

Light microscopy has shown that approximately 24 hours after MeA training, there was an increase in dendritic spine density in both left and right IMHV, with the largest increase being observed in the left IMHV. This change was accompanied by an increase in measures of spine head diameter and a decrease in spine stem length of MeA birds (Patel and Stewart, 1988). Transient transcranial electroshock showed that this increase in spine density was due to formation of a memory for the MeA bead because amnesic MeA-trained birds

exhibited similar spine densities to Water trained birds (Patel et al., 1988). Some of these learning related synaptic changes are abolished by anisomycin (Bradley and Galal, 1987).

As some birds may have had greater recall of the Water experience than MeA birds did of their experience, this may explain why Water-training resulted in greater changes than MeA-training in some regions. In addition, it may be erroneous to assume that MeA training is a "stronger stimulus" than Water training. Since the two training experiences are different, it maybe that one is "more" reliant upon one (or more) aspects of the training paradigm than the other. MeA training is an aversive memory stimulus whereas Water training could be a stimulus for appetitive memory formation. Hence, one of the training experiences could evoke more dramatic changes in an area that is more receptive to that memory aspect, thus explaining why the Water experience created greater morphological alternation in some areas compared to others.

IMMEDIATE-EARLY GENES FOS AND JUN

Most Immediate-early genes (IEGs) predominantly encode for nuclear proteins, leading to the belief that such proteins might serve as transcription factors, activating late gene expression (Setoyama et al., 1986; Bohmann et al., 1987; Angel et al., 1988). These transcription factors cause late gene activation/repression by binding to their corresponding binding-site in the regulatory region of late genes. Late genes encode for many proteins within the cell. These include structural proteins and enzymes. The structure of many transcription factors are already known, but this appears to be the tip of the iceberg, with more transcription factors being regularly identified. The exact transcription factor complex that is ultimately produced is a function of both the cell type and the stimulus.

IEGs share several distinct features:

(a) they are generally expressed at very low levels in non-stimulated cells;

(b) they are rapidly induced by cellular stimuli;

(c) their transcriptional induction is very transient (30 - 60 minutes) and independent of new protein synthesis,

(d) subsequent transcriptional termination requires new protein synthesis; and

(e) their corresponding mRNAs are rapidly degraded (Armstrong and Montminy, 1993).

It is thought that these viral-IEG (v-*onc*) evolved from mutations derived from cellular-IEGs (c-*onc*; Bishop & Varmus, 1982). In the nervous system these transcription factors are considered to stimulate late gene expression in response to neuronal signalling (Christry et al., 1988; Curran and Franza, 1988; Milbrandt, 1987). Geolet et al (1986) suggests that they act as a third messenger system by translating cellular information into biochemical changes. For simplicity the format c-*onc* and Onc are employed throughout this thesis when refering to gene mRNA and protein product respectively; onc representing any oncogene eg. fos or jun.

V-*fos* is the viral gene that causes bone cancer in fibroblast osteosarcoma (v-*fos*; Finkel et al., 1966; Finkel & Biskis, 1968). Its cellular counterpart c-*fos* was later found to occur naturally in the genomes of animals (Ransone and Verma, 1990). Researchers later identified another oncogene, which they named c-*jun* after the Japanese word for 17, presumably as it was the 17th gene studied (Maki et al., 1987).

The Fos and Jun protein families are located exclusively in the nucleus (Sambrucetti and Curran, 1986). C-*fos* and c-*jun* are expressed in response to many stimuli in the brain. Examples of stimuli include cerebral ischemia,

induced seizures, neuronal bursting, hormones, nocioceptive input, nerve growth factors (NGF), light and depolarising ions (An et al., 1993; Uemura et al., 1991; Dragunow and Robertson, 1988; Dragunow et al., 1989a; 1989b; Curran et al., 1985; Bullitt, 1990; Hunt et al., 1987; Meyer et al., 1993; Nir and Agarwal, 1993; Curran and Morgan, 1986).

Initially there appears to be some conflicting views regarding the induction of c-*fos* (mRNA). Morgan and Curran (1991) are of the opinion that c-*fos* induction is an all or nothing phonemea, whereas Presley et al (1990) have found that the number of neurones expressing c-*fos* is proportional to the intensity and duration of stimulation. It may be however, that each cell has its own threshold for induction which is a function of individual cells so both groups are correct. A refractory period of several hours exists after initial stimulation where it is impossible to re-induce c-*fos* (Morgan et al., 1987). On the other hand c-*jun* is constitutively expressed (Bading et al., 1991).

There are at least 4 different members of the "Fos family" (*c-fos, fos-B, fra-1, fra-2*) and the protein product of each of them (c-Fos; Fos-B, Zerial et al., 1989; Fra-1, Cohen and Curran, 1988; Fra-2, Nishina et al., 1990) may interact with any of the proteins of the "Jun family" (c-Jun; Jun-B, Ryder et al., 1988; Jun-D; Hirai et al., 1989) in order to produce dimers acting as activator proteins-1 (AP-1s; Morgan and Curran, 1991). The different members of Fos and Jun gene families each have their own kinetics of induction. For example the Fras and JunB have much longer times of induction and subsequent breakdown compared to Fos or Jun (Bartel et al., 1989). Only members of the Jun family can form homodimers (Halazonetis et al., 1988). It appears that Fos (and other family members) serves to modulate the activity of Jun because Jun (and family), and not Fos, has the ability to bind directly to DNA sequences (Ransone and Verma, 1990). In fact, Sonneberg et al (1989a) suggest that Jun may be the major component of transcription factor AP-1 whereas Fos and

Fras are variable components. These AP-1 complexes can then bind to their corresponding AP-1 binding site, so initiating or repressing induction of these genes (Lee et al., 1987). Late genes that are known to be regulated by AP-1 include the proenkephalin gene (Sonnenberg et al., 1989c)

LTP and IEG induction

In its role as a model for synaptic plasticity, LTP and the induction of c-*fos* and c-*jun* have been studied with some conflicting results. These contradictions could be due to differences in the methods employed for LTP induction. In general, robust c-*fos* expression was observed when LTP lasting longer than a few hours (long-lasting-LTP, LL LTP) was elicited by a single stimulation session in freely moving rats (Dragunow et al., 1989b; Jeffery et al., 1990; Nikolaev et al., 1991). On the other hand, no reproducible c-*fos* expression was found when LTP was induced under conditions in which a single stimulation session apparently did not produce LL LTP (Douglas et al., 1988; Cole et al., 1989; Wisden et al., 1990).

Expression of Jun family members has been found to correlate weakly if at all with LTP formation (Cole et al., 1989; Wisden et al., 1990) in experiments performed on anaesthetized animals with no demonstration of LL LTP. LTP induction in freely moving rats does not induce c-*jun* mRNA (Kaczmarek, 1993). However, the expression of another IEG, *zif*/268 (also known as NGFI-A, *Krox* 24 and *Erg*-1) seems to be more reliably and easily induced as a result of LTP (Cole et al., 1989; Wisden et al., 1990). In fact, a correlation between the level of LTP induction and the level of *zif*/268 expression has been noted (Richardson et al., 1992). And recently Abrahams et al (1993) have tentatively shown that *Zif*/268 is associated with LTP_2 and LTP_3 whereas a weak correlation exists between Fos and Jun (and other family members) induction in LTP_3 . This has lead to their suggestion that *Zif*/268 is involved in the

initiation, whereas Fos and Jun contributes to the stabilization or maintenance of LTP_3

Arachidonic Acid and IEG induction

Activation of the enzyme PLA_2 by an influx of Ca^{2+}, presumably via NMDA receptor activation, causes the release of free arachadonic acid. Arachidonic acid can be metabolised by three different routes; the lipoxygenase, cyclooxygenase or epoxygenase pathways. Haliday et al (1991) found that arachidonic acid and its lipoxygenase metabolites induced c-*fos* mRNA in adipogenic TA1 cells, whereas other fatty aids such as oleic or linoleic acid did not. They also showed arachidonic acid was not required for c-*jun* induction. The synthesis of lipoxygenase metabolites such as 12-hydroperoxyeicosatetraenoic acid (12-HPETE) and its subsequent metabolism have been shown to be important for Phe-Met-Arg-Phe amide (FMRFamide) - induced presynaptic inhibition of sensory cells (Piomelli et al., 1987) as well as the response to L14 motor neurones to histamine in *Aplysia* (Piomelli et al., 1988; 1989); both responses are presumably mediated by the opening of a specific class of K^+ channels (S channels).

Arachidonic acid metabolised via the cyclooxygenase pathway can lead to increased cyclic AMP (cAMP) levels. cAMP is also known to be involved in the induction of c-*fos* and c-*jun* (see later). The role, if any, that the epoxygenase pathway plays in c-*fos* or c-*jun* induction remains to be determined.

In the context of passive avoidance learning in the chick, arachidonic acid metabolism may play a role in c-*fos* induction because injection of the inhibitors of PLA_2 renders the chick amnestic (Hölscher and Rose, 1994). Conversely, intracranial injections of arachidonic acid enhance recall for the task when chicks are trained on the weaker stimulus of 10% MeA (Serrano et al., 1991). Whether arachidonic acid is indirectly inducing c-*fos* and c-*jun* in

this paradigm remains to be shown. The results from LTP involving release of arachidonic acid suggest that the enzyme PLA_2 is only active during the initial stages of LTP to produce free arachidonic acid and later on (after 45 minutes) PLC is responsible for arachidonic release. So, in the two waves of neuronal activity already described, arachidonic acid could be initially functioning as a retrograde messenger and an inducer of IEGs, and in a second later phase of neuronal activity as an IEG inducer.

Receptor Activation and IEG induction

At least two types of glutamate receptors appear to induce IEG expression. Activation of the NMDA ionotropic receptor has been shown to induce c-*fos* and c-*jun* and their proteins. Antagonists of this receptor result in inhibition of c-*fos* and c-*jun* induction, the other receptor being the exclusively ionotropic, kainate receptor (K). Since the kinetics of IEG induction is prolonged in the K- compared to the NMDA- receptor it has been suggested that both receptor subtypes may employ different second-messenger pathways to transmit their signals (Sonnenberg et al., 1989a).

Metabotropic receptors are linked to G-proteins. Their activity conveys information of receptor activation across the cell membrane. Activation of metabotropic receptor can culminate in either the release of cAMP which activates a cAMP dependent kinase (PKA), or the release of the second messengers DAG and inositol-trisphosphate (IP_3) via activation of PLC. DAG can directly activate PKC which in turn can mediate c-*fos* induction (Gilman, 1988; Greenberg et al., 1986). Generation of IP_3 can lead to liberation of Ca^{2+} from internal stores. Calcium may then bind to calmodulin and activate the Ca^{2+}/calmodulin-dependent kinase (CAM-kinase). Indeed, CAM-kinase activity is necessary for the induction of c-*fos* in response to depolarisation of PC12 cells (Morgan and Curran, 1986). However, since only general inhibitors

of CAM kinases were used, one cannot be sure which species of CAM-kinase (type I and/or II) was involved. At present the nicotinic acetylcholine and the NMDA/glutamate receptors are the only ionotropic receptors implicated in the regulation of gene expression. The nicotinic acetylcholine receptor gates the entry of Na^+ whilst the NMDA receptor gates Ca^{2+} into the cell. However, ultimately, both receptors may be stimulating IEG induction via Ca^{2+} dependent mechanisms (Morgan and Curran, 1986). The increase in intracellular Ca^{2+} can then bind to CAM-kinase as Ca^{2+} does from internal stores.

Cyclic-AMP has been implicated in the expression of genes in the nervous system (Eiden et al., 1984). All of the known cellular effects of cAMP occur via the catalytic subunit of PKA, which suggests that cAMP might correspondingly regulate transcription through the reversible phosphorylation of transcription factors (Armstrong and Montminy, 1993). The promoter of specific genes contains a cAMP responsive element (CRE) sequence. This sequence binds the CRE-binding protein (CREB) and is both necessary and sufficient for cAMP inducibility (Montminy et al., 1986; Montminy and Bielezikjian, 1987). CRE-binding does not appear to be the only factor capable of mediating transcriptional response to cAMP. Dwarki et al (1990; Yamamoto et al., 1988) has shown that both Fos and Jun are incapable of dimerising with CREB. However, other workers disagree with these findings (Szekely et al., 1993). Macgegor et al (1990) have shown that Jun is capable of binding to CREB protein CRE-BP1 (Maekawa et al., 1989). But, this complex can only bind to the CRE and not to the AP-1 binding site. Nevertheless, Fos and Jun are incapable of binding to the same CREB complex. Proteins such as ATF-1 (a relative of CREB; Rehfuss et al., 1991) and JunD (Kobierski et al., 1991) can also stimulate transcription in a cAMP-dependent manner.

Activation of transcription in a Ca^{2+}-dependent manner has been shown in many neuronal systems. Both Ca^{2+} and cAMP responses may converge on a single calcium/cyclic AMP response element (CaRE/CRE) (Sheng et al., 1990). CREB can be directly phosphorylated by the Ca^{2+} -dependent CAM-kinases *in vitro,* suggesting that the Ca^{2+} and cAMP pathways converge on the same regulatory protein through distinct kinases. Sheng et al (1991) further proposed that Ca^{2+} could mediate CREB activity in a PKA-independent manner. However other workers (eg. Ginty et al., 1991) disagree. Ginty et al (1991) found that induction can only occur in a Ca^{2+} PKA-dependent manner. Different experimental procedures coupled with different cell lines could account for these discrepancies. In addition, another Ca^{2+} binding site that is distinct from the CaRE/CRE site has been identified (Sheng et al., 1990). It is of interest that Chetkovich and Sweatt (1993) have demonstrated that activation of the NMDA receptor in LTP in hippocampus CA1 slices leads to increased cAMP via adenylate cyclase. Further work into the exact role (if any) that cAMP or Ca^{2+} plays in c-*fos* and c-*jun* learning-related expression is necessary.

A rise in intracellular Ca^{2+} is also known to cause the release of nitric oxide (NO). Peunova and Enikolopov (1993) found that stimulation of PC12 cells required both NO and Ca^{2+} for c-*fos* and c-*jun* induction. NO on its own was insufficient to provoke IEG induction but in combination with Ca^{2+} it increases c-*fos* and c-*jun* induction above that normally induced by Ca^{2+} alone. This combination also increased the binding activity of AP-1. NO was considered to act via the G-protein and PKA-CREB-CRE system. NO has been shown to induce c-*fos* and *jun*B, but not c-*jun* or *jun*D, and increase AP-1 binding activity after activation of the cyclic GMP pathway in PC12 cell (Haby et al., 1994). In this case, GMP is thought to activate PKG which can then phosphorylate CREB. Alternatively, PKG may affect gene expression more indirectly by phosphorylating a protein that leads to protein phosphatase inhibition. In the chick passive avoidance training task, 30 minutes after

training, intracellular levels of Ca^{2+} are raised in the left IMHV and intracerebral injections in this region of an inhibitor of NO or Ca^{2+} uptake produces amnesia (Hölscher and Rose, 1992; 1993; Clements and Rose, 1994). Whether this results in c-*fos* and c-*jun* induction in the presynaptic neurone remains to be determined.

The role of PKC in transcriptional activation is not well understood. It appears that it stimulates induction via a dyad symmetry element (DSE; also referred to as serum responsive element, SRE). IEG induction via the PKC pathway has been shown to lead to c-*fos*, c-*jun* and *jun*B expression (Bartel et al., 1989). Its role in learning and memory-related IEG expression is yet to be determined. However, it is known that once translated Fos undergoes rapid modification. This is mainly due to phosphorylation of the serine and theronine residues (Morgan and Curran, 1986). Phosphorylation of AP-1s by PKC is also known to increase their binding affinity (Morgan and Curran, 1986).

It is of interest that Bullock et al (1993) showed that there was an increase in the level of inositol phosphate (IP) after stimulation of the glutamate receptors by AMPA or quisqualate in the right IMHV. This induction was not attenuated by MK-801, suggesting IP turnover via the non-NMDA receptors in the right IMHV. In addition, Clements and Roses' (1994) presynaptic Ca^{2+} studies had similar results, suggesting that activity in the right IMHV stimulates a glutamate receptor-operated second messenger system. Hence, the time for IEG induction is slightly longer in the right than the left IMHV and, in the time window for passive avoidance memory formation, the right IMHV could act as a relay station in the memory cascade.

Each transduction system has different kinetics of c-*fos* and c-*jun* induction, thus providing a mechanism where genes can be activated at different times

relative to stimulation. Since it appears that memory formation is spatial and temporal, involving both pre- and post- synaptic modifications, it maybe that all these systems for gene activation (and repression) are active but at different times and brain regions in the memory cascade. A common element in IEG induction systems seems to be Ca^{2+}, so depending on the stage in the memory cascade a different "relay system" for gene activation maybe in action.

AIMS

The basic aims of this thesis pertaining to passive avoidance memory formation are five-fold:

(a) To study the molecular biology of c-*fos* and c-*jun* induction.

(b) To determine which species of Fos, Jun and their related proteins are expressed.

(c) To create a spatial and temporal map of Fos and Jun activity.

(d) To determine whether activation of the NMDA receptor leads to Fos and Jun induction in the memory cascade.

(e) To elucidate the time course of protein synthesis following passive avoidance learning and compare this with the data available for glycoprotein synthesis.

CHAPTER 2

Induction and translation of c-*fos* and c-*jun* mRNA

INTRODUCTION

The measurement of c-*fos* (or c-*jun*) mRNA induction is a useful marker for showing active neurones/regions of the brain and spinal cord (Sagar et al, 1988). Previously the uptake of radio-labelled 2-deoxyglucose (2-DG) was used as an indication of neuronal activity. An increase in neuronal metabolism and hence glucose requirement is reflected by increased uptake of 2-DG (Sokoloff et al,1977). However, there are some discrepancies between the results obtained by the c-*fos* and 2-DG mapping methods. Metrazole, which induces general seizures, leads to an enhanced 2-DG uptake in the cerebellum and substantia nigra (as well as other brain regions, Ben-Ari et al., 1981) of the rat. But metrazole induced-seizures did not elevate Fos protein in these two brain regions (Dragunow and Robertson, 1988; Morgan et al., 1987; although c-*fos* mRNA was induced in the cerebellum). C-*fos* was shown to be induced in the IMHV of the chick (and hippocampus, medial neostriatum, paleostriatum augmentatum, stratum opticum, cerebellum) previously not "high-lighted" by 2-DG studies (Abramova et al., 1992) after imprinting. These mismatches could have been due to 2-DG detecting dendritic and axonal activity whereas c-*fos* is confined to the nucleus and is therefore only a measure of nuclear activity (Dragunow and Faull, 1989). The major advantage of using c-*fos* activation as a measure of neuronal activity compared with 2-DG uptake is that induction of c-*fos* is transient and rapid in response to stimuli. In comparison, glucose is continually required by the cell, hence 2-DG uptake is constant and any activity-dependent increase would be over and above this basal activity, providing a less sensitive method. Moreover, measuring c-*fos* induction

affords a non-invasive method of measuring increased neuronal activity. C-*fos* is also considered to play a role in the memory formation cascade and is therefore a reaction to the stimulus; whereas 2-DG reflects an increase in metabolic activity and hence is a very general phenomenon, imparting less useful information.

2-DG uptake studies in the chick following passive avoidance training have demonstrated that the IMHV, PA and LPO showed enhanced activity 30 minutes after training (Kossut and Rose, 1984). Rose and Csillag (1985) expanded these observations to show that more uptake occured in the left IMHV and left LPO compared to the contralateral regions. After measuring induction of c-*fos* mRNA by Northern blot analysis, Anokhin et al (1991) showed that c-*fos* was induced in the right IMHV and both LPOs 30 minutes after passive avoidance training. In another experiment, where Anokhin and Rose (1990) used a non-aversive learning paradigm, the pebble floor, they demonstrated that c-*fos* and c-*jun* mRNA were induced after the learning experience. The location of c-*fos* and c-*jun* was very widely distributed throughout the whole forebrain.

Hunt et al (1992) have shown that after pain infliction in the rat by exposing its leg to a hot plate, induction of c-*jun* mRNA was observed in the spinal cord but no members of the Jun family of proteins were subsequently expressed. In another model, the gerbil, both c-*fos* and c-*jun* mRNA were induced in the hippocampus in response to transient global ischemia but again no Fos or Jun proteins were later detected (Kiessling etal., 1993). Moreover in an example, already noted above, metrazole induced-seizures also provided some conflicting data regarding c-*fos* mRNA and its subsequent translation. Metrazole induced c-*fos* mRNA in the rat's cerebellum 30 minutes after stimulation but Fos protein was not detected 2 hours later (Dragunow and Robertson, 1988; Morgan et al., 1987).

There are several factors that regulate mRNA stability in vivo. RNA polymerase II transcripts in the nucleus are known as heterogeneous nuclear RNA (hnRNA). A large proportion of these are modified (eg. have their introns removed) forming mRNA. The 5′ end is capped by the addition of a methylated G neucleotide. This 5′ cap plays a role in the initiation of protein synthesis and also appears to protect the growing RNA transcript from degragation. Poly(A) can also be added to the 3′ end. The greater the number of A nucleotides (up to 30 nuclotides) added the more resistant to degragation the mRNA is (Atwater et al., 1990). Obviously if these modifications were not carried out then the mRNA would be degraded before translation could occur. Especially since immediately early genes are only transliently transcibed. This provides an explaination for the lack of Fos or Jun protein observed, even when the mRNAs are abundant.

This chapter aims to test whether, under different conditions of stress experienced by the chick, all the c-*fos* or c-*jun* mRNA induced is, in fact, translated.

METHODS

NORTHERN BLOTTING PROTOCOL

The technique of northern blotting was employed to identify c-*fos* and c-*jun* mRNA. Northern blotting involves the separation by gel electrophoresis of a relatively pure aliquot of the total RNA. After transfer on to a nitrocellulose membrane (blotted), the mRNA of interest is identified by hybridisation with a corresponding radioisotope labelled cDNA probe. Exposure to a photographic film allows identification of the c-*fos* or c-*jun* band.

The Northern blotting assay was set-up using metrazole, which is known to induce c-*fos* or c-*jun* maximally (Morgan et al., 1987). Since only Jun and Fos

proteins, and not their mRNAs, are capable of late gene activation, to prevent repetition, the role that metrazole plays in IEG induction is discussed in greater detail in chapter 3.

Total RNA extraction

RNA was extracted using an adaptation of the guanidium thiocyanate-phenol-chloroform extraction method of Chomczynski and Sacchi (1987). To prevent RNA degradation due to endogenous RNases, the tissue samples were kept frozen on solid CO_2 until required. Since the total amount of tissue collected was on average only 60mg the following extraction was performed in 1.5ml eppendorf tubes. First the tissue was macerated at room temperature in 550µl of solution D (4M guanidium thiocyanate, 25mM tri-sodium citrate, 0.5% sarcosyl, 0.1M 2-mercaptoethanol). To acidify the reaction conditions, 2M sodium acetate pH 4 (0.2 M) was added to the homogenate. Protein was removed by the addition of an equal volume of water-saturated phenol. These phenol-protein complexes were removed by adding chloroform (98% chloroform, 2% iso-amyl-alcohol) so that the ratio of chloroform to phenol was 1:5 (v:v). The mixture was left on ice for 15 minutes. The sample was then centrifuged at 15,000g for 10 minutes. This yielded three layers, the top layer containing RNA, a thin protein-rich layer and, at the bottom, a layer that contained phenol and chloroform. The RNA-containing layer was collected in a fresh tube and an equal volume of isopropanol added. This was then left at -20°C for at least 1 hour (but no longer than 2 hours). The sample was centrifuged as before. The pellet containing RNA was resuspended in 110µl of solution D, 11µl of 2M sodium acetate, 303µl of 70% ethanol, and left at -20°C for 1 hour. After centrifuging again the pellet was washed in ethanol. At this point the pellet contained contaminating polysaccharides which were removed by leaving overnight at -20°C in 50µl of autoclaved 'sterile water' (deionized, filtered and containing DPEC; an RNase inhibitor), plus 2.5

volumes of 70% ethanol, and 0.1 volume 3M sodium acetate pH 5.2. Further centrifugation yielded a pellet that was again washed in 70 % ethanol, centrifuged for another 2 minutes and dried in a vacuum desiccator for 15 minutes (25 lb/sq inch). The amount of isolated RNA was quantified by dissolving the dried pellet in 50µl of 'sterile water'. 5µl of this was added to 1.5µl of water and its absorbance read at 260nm. To determine the amount of protein (and phenol) contamination, the absorbance at 280nm was also noted. This method should yield a sample that has a ratio of RNA: Protein of approximately 2 (A_{260}:A_{280}). If the ratio was less than 1 the sample was too heavily contaminated with protein to be used. Since 1 absorbance unit is equivalent to 40mg ml^{-1} the amount of RNA per sample could be calculated. The RNA was stored at -20°C with 2.5 volume 70% ethanol, and 0.1 volume 3M sodium acetate as previously described.

Separation of total RNA

The RNA was denatured to make it single stranded and to prevent secondary structures forming and thus deviating from a linear relationship between distance travelled in gel and molecular weight. 15µg of RNA was incubated at 65°C for 15 minutes with 35µl of denaturing buffer (50% deionised formamide, 2.2M formaldehyde, 1x running buffer; MOPs {1x MOPs = M 3-(N-Morpholino) propane-sulphonic acid, 10mM sodium acetate, and 1mM EDTA}, 0.015% bromophenol blue, 0.015% xylene cyanol, 1.5% ficoll, and 20M ethidium bromide). Samples were loaded onto a horizontal 1.0% agarose/2.2M formaldehyde gel and electrophoresed in MOPs buffer for 2-3 hours at 70-80 volts. The separation was monitored as the denaturing buffer yielded two bands; one that ran just after the highest molecular weight RNA (xylene cyanol) and a second one which ran slightly ahead of the lowest molecular weight RNA (bromophenol blue). Since ethidium bromide binds to the RNA, RNA degradation could be visualised by Ultra Violet light.

Transfer of RNA on to a membrane

After electrophoresis, the gel was washed twice in 10x SSC (1x SSC is 0.15M sodium chloride, 0.015M sodium citrate, pH 7.2), each wash lasting 10 minutes. The membrane (Hybond-N, Amersham) was activated by boiling for 10 minutes in sterile water. Three pieces of 3M and approximately 4 inches in depth of 1M filter paper were cut to the size of the gel. Two pieces of the 3M filter paper were then soaked in 10x SSC and the other piece in 2x SSC. A cloth soaked in 10x SSC was placed over a piece of glass so that it dipped into a bath of 10x SSC. The filter paper soaked in 10x SSC was placed on top of this cloth, followed by the inverted gel. Next the nitrocellulose membrane was placed on top of the gel. Since the movement of salt ion from the SSC bath carried the negatively charged RNA from gel to membrane it was important that the salt

solution only moves up through the centre of the gel, hence the sides of the gel had parafilm strips laid on them. The 3M filter paper, soaked in 2x SSC was placed on top of the membrane (to start the salt gradient), and above this a mound of dry 1M filter paper. The whole assembly was evenly weighed down with a weighted glass plate and left overnight at room temperature. To monitor the blotting process, one could still view the ethidium bromide bound RNA under UV light on both gel and membrane. RNA was then permanently bound to membrane by exposing it to UV light for 2.5 minutes. The blot was then stored ready for hybridisation.

Excision of c-fos probe from its plasmid

The probe, *Pvu*II-*Pvu*II 508-bp fragment of the exon 4 of chicken c-*fos* gene (provided by Dr S. Kawai; for sequence see Fujiwara et al., 1987), was maintained and amplified in pGEM plasmid. Amplification of the plasmid was carried out in transformed *E. Coli* cells. 0.5ml of plasmid solution was added to 100ml of *E. Coli* (60 r) and left on ice for 40 minutes. An aliquot of this *E. Coli* solution was spread over the surface of an agar plate. This was left to multiply at 37°C for 24 hours. One colony of *E. Coli* was then mixed with 2mls of sterile LB broth (1% bactrotrypton w/v, 0.5% yeast extract w/v, 0.5% NaCl w/v, pH 7-7.6), and this was again left to stand for 12 hours at 37°C. Fifty percent of this solution was then mixed with glycerol to a final glycerol concentration of between 15-25%. Each sample was stored at -70°C until required.

500µl of the remaining *E. Coli* solution was poured into 100ml of LB broth containing ampicillin (100mg/ml), so that only the *E. Coli* cells containing the c-*fos* probe, and hence the ampicillin resistant gene, would survive and replicate. This was incubated at 37°C for between 12-18 hours. The cells were separated from the broth by centrifuging at 10,000g for 10 minutes. They were

then lysed by resuspending in 5ml of solution 1 (50mM glucose, 25mM Tris-HCl, 10mM EDTA, 0.5mg/ml of lysozyme) and left on ice for 15-30 minutes, followed by the addition of 10ml of solution 2 (0.2N NaOH, 1% SDS), and after 2-5 minutes 7.5ml of solution 3 (5M Potassium acetate) and left for 30 minutes on ice. The cell debris was separated from the plasmid by ultracentrifugation (15,000g for 1 hour). The supernatent now contained the required plasmid. Other unwanted nucleic acids were removed by precipitation in 0.6vol isopropanol (-20°C for 30 minutes). The pellet was washed in ethanol, dried and suspended in 500µl of water and 1ml of 7.5 M ammonium acetate and left at -20°C for 30 minutes. The resulting mixture was spun at 12,000g for 10 minutes and the plasmid containing supernatant was incubated for 30 minutes in isopropanol. This time the dried pellet was resuspended in 1ml of water, 1ml of 11.2% 8,000 PEG, 0.7M NaCl solution and left on ice for 30 minutes. After centrifugation the supernatent again had an equal volume of the PEG/NaCl solution added. The plasmid containing pellet was washed 3 times in ethanol to remove any traces of PEG and thoroughly dried. The plasmid was resuspended in water ready for probe removal.

To remove the c-*fos* probe from its plasmid, restriction enzymes were employed. The c-*fos* probe was located between a site recognised by ECoR1 and another site recognised by BamH 1. However these enzymes function optimally under different conditions, and hence two sequential reactions were necessary to completely remove c-*fos* probe from its plasmid. Firstly 30µg of plasmid DNA was incubated with 150 units of BamH1 (in 20mM Tris-HCl, pH 7.8, 7mM $MgCl_2$, 100mM NaCl, 2mM 2-mercaptoethanol, 0.1mg/ml BSA) at 37°C for 1 hour. Next 150 units of EcoR 1 (in 10mM Tris-HCl, pH 7.5, 10mM $MgCl_2$, 100mM NaCl, 1mM 2-mercaptoethanol, 0.1mg/ml BSA) was added and a further incubation of 1 hour carried out. An aliquot of the sample was then separated by running on a non reducing, low melting point, agarose gel as stated before. A band that ran at approximately 500 bp was cut out from the gel.

This band corresponded to c-*fos* probe and was separated from the agarose by adding 2-3 volumes of STE (10nM Tris-HCl pH 7.6, 100μM EDTA, 0.1% SDS) and heating up to 65°C for 10 minutes, so that the agarose melted. Next an equal amount of phenol (stored under Tris pH 8) was added and the mixture spun for 5 minutes. The addition of $^1/_2$ volume phenol and $^1/_2$ volume of chloroform then removed any traces of phenol after centrifugation. The pellet was further purified by a salt precipitation (0.1 volumes 3M NaAc, pH 5; 2.5 volumes ethanol) overnight at -20°C. The dried pellet was resuspended in water ready for radioisotope labelling.

Isotopic labelling of the c-fos and c-jun probes

This was carried out using the technique of nick-translation where adenosine residues in the DNA of the *Pvu*II-*Pvu*II 508-bp fragment of the exon 4 of chicken c-*fos* gene (or c-*jun* probe) was replaced with ^{32}P-labelled adenosine. This was achieved by incubating the probe (c-*fos*) with ^{32}P-dATP (deoxy-adenosine triphosphate), the other three unlabelled dNTP$_s$ (deoxy-nucleotide triphosphate), and the enzyme DNA polymerase 1 (DNA pol 1). In this reaction DNA pol 1 had a dual action. Firstly, it added nucleotide residues to the 3' hydroxyl terminus that was created when one strand of double-stranded DNA was nicked. Secondly, it also had a 5' to 3' exonucleotidase activity, hence it removed the nucleotide from the 5' end. The removal of this nucleotide exposed a 3' site to which DNA pol 1 added a nucleotide. This sequential addition resulted in movement of the nick (nick translation) along the DNA molecule. If all four nucleotides were not present then the reaction would proceed extremely slowly, if at all. For economic reasons only one dNTP was labelled and its concentration was far less than the unlabelled dNTPs' individual concentrations.

The reaction was carried out using the Amersham Nick Translation Kit. The following reagents were added to a sterile vessel: 100µg C-*fos* (or c-*jun*) probe, 6nMols dCTP, dGTP, dTTP, buffer, 100µCi ^{32}P-dATP, sterile water, and 5U DNA pol 1. This reaction was left at 15°C for approximately 1 hour. It was not carried out at a higher temperature because considerable amounts of 'snap back' DNA can be generated by DNA pol 1 copying the newly synthesised strand. Before the reaction was terminated, the percentage incorporation of ^{32}P was determined by taking 2x 1 µl aliquots of the reaction solution and drying it on 2 pieces of DE-81 filter paper. One filter was washed with 5% TCA. This removes any small molecules such as nucleotides, leaving only the probe on the filter paper. The activity of the washed and unwashed filters was determined by scintillation counting.

Percent incorporation of ^{32}P was calculated ({cpm washed filter/cpm unwashed filter} x 100). The monitoring continued if necessary until an incorporation of 40-60% was achieved. To terminate the reaction the mixture was applied to a sterile 5ml sephadex (G-50) column, equilibrated in TES buffer (10nM Tris-HCL pH 7.6, 100µM EDTA, 0.1% SDS). 200µl aliquots of the eluent were collected and the activity of a portion of each sample was quantified by scintillation counting. The first peak of activity was labelled probe and the second unincorporated ^{32}P-dATP. The aliquots that constituted the labelled probe were then pooled and denatured by alkaline hydrolysis; for every 100µl of eluent 3µl of 10M sodium hydroxide was added and left at 37°C for 5-10 minutes. The solution was neutralised using concentrated hydrochloric acid.

Identification of mRNA from isolated total RNA

The c-*fos* (or c-*jun*) probe hybridised to the complementary bases of the c-*fos* (or c-*jun*) mRNA, which was bound to the membrane. To increase sensitivity, and decrease the background, so that the signal to noise ratio was optimised,

this assay included the addition of sheared salmon sperm DNA. This competed with the probe for sites of non-specific binding, that is where different species of RNA share regions of homology with the probe's base sequence. Also the salmon sperm DNA was in excess to the probe; hence, in theory, the probe could bind only to the c-*fos* (or c-*jun*) mRNA. Formamide decreased the hybridisation temperature, which was a function of the RNA-probe under investigation. A more manageable temperature of 37°C also meant that proteins would not be denatured. The salt concentration was also critical because at low ionic strength nucleic acids hybridise very slowly, but as ionic strength increases, the reaction rate increases. However, as the salt concentration increases so does the stability of mismatched duplexes, hence an optimal concentration had to be found.

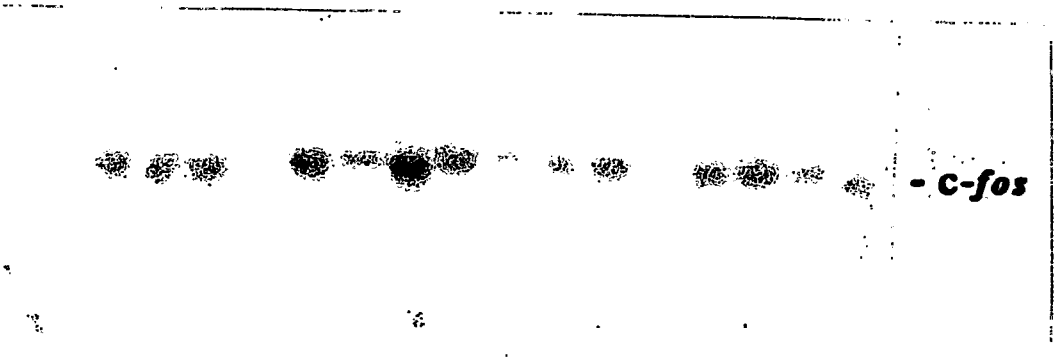
The membrane was heat sealed in a plastic bag containing hybridisation buffer (5x SSC, 0.1% ficoll, 0.1% polyvinyl pyrrolidone, 0.1% bovine serum albumin, 0.5% SDS, 5mM EDTA, 250mg/ml salmon sperm DNA, 100mg/ml tRNA, 50% formamide, 30nM phosphate buffered saline) and left to incubate at 37°C for 3 hours. The hybridisation buffer was then replaced with a fresh batch which contained the labelled c-*fos* (or c-*jun*) probe. It was left to incubate as before at 37°C for 16 hours. The membrane was washed in 2x SSC for 5 minutes at room temperature and twice for 30 minutes in 2x SSC containing 0.2% SDS, at 65°C. As reprobing, or reuse of the membrane may be required, it was wrapped in cling film to prevent drying out.

Autoradiography

Autoradiography allowed visualisation of the c-*fos* (or c-*jun*) band on the membrane. The membrane was placed next to a photographic film (Amersham Hyperfilm MP) inside a light proof cassette. After 3-4 days at -80°C the film was developed (4 minutes developer, 10 minutes fixative, 20 minutes

water). The intensity of c-*fos* bands was quantified using a NSCA gel reader; the level of c-*fos* or c-*jun* mRNA induced was directly proportional to the intensity of the observed band. Film linearity of response was monitored by apposing for different time periods. For each film the intensity of bands derived from stimulated birds were compared to the intensity of Quiet (unstimulated birds) bands. These results were expressed as percentage induction. As long as the films response was linear (ie. not over or under exposed) the percent induction for each test band should be approximately the same for each film.

Figure 2.1 shows representative autoradiograms after Northern blotting for c-*fos* (a) and c-*jun* (b) mRNA.



(a) A Northern blot autoradiogram after probing for c-*fos* mRNA (random samples were taken from expt. 2.2.)

(b) A Northern blot autoradiogram after probing for c-*jun* mRNA (random samples were taken from expt. 2.2.)

Figure 2.2.

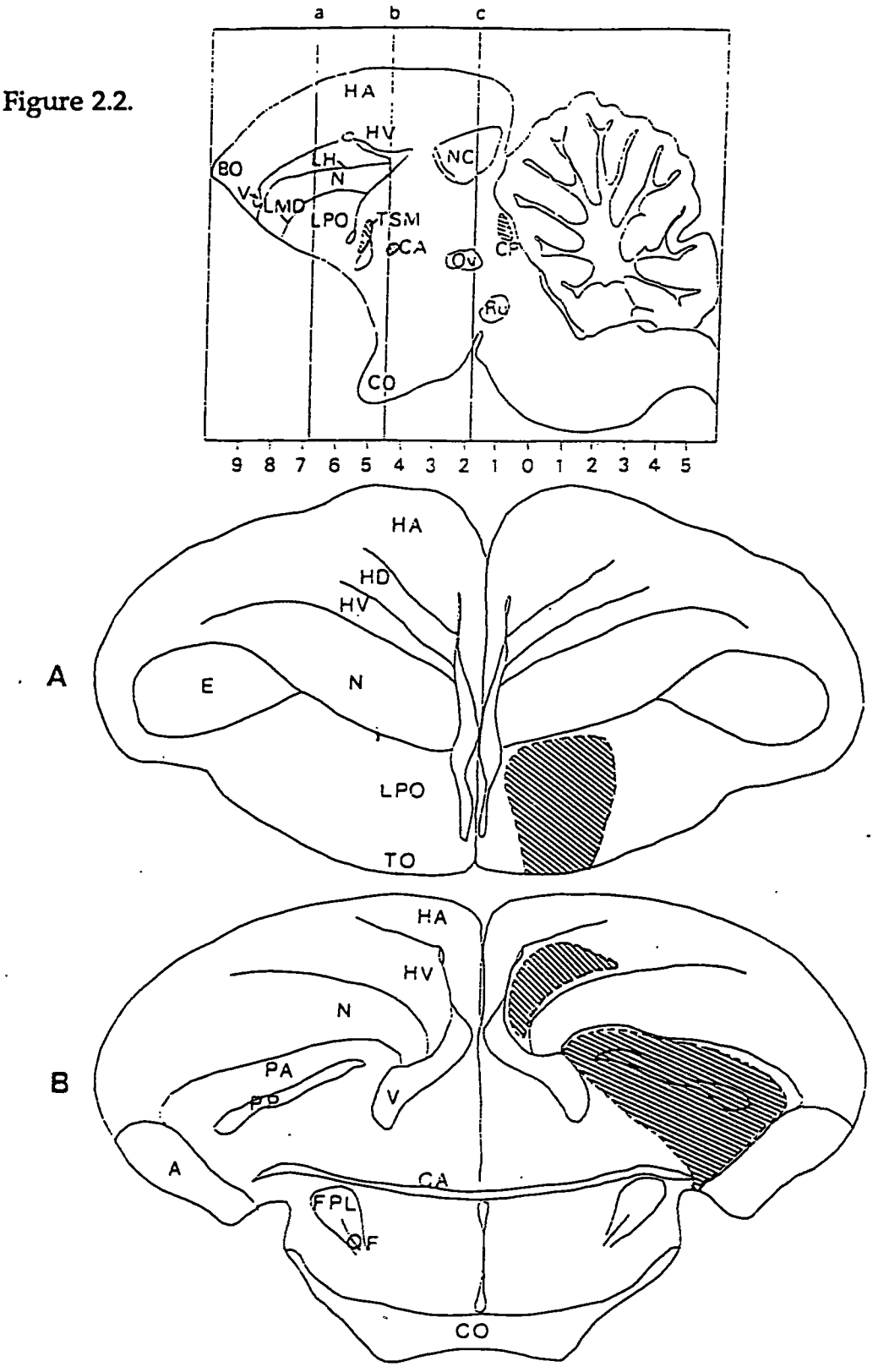


FIG. 1. Schematic drawings of coronal slices of day-old chick brains prepared using a brain mould. The position and angle of cuts are shown in the sagittal scheme (stereotaxic coordinates after Youngren and Phillips, 1978). The first slice, represented by face A (taken at level "a"), was used for dissecting "LPO," and the second one, represented by face B (taken at level "b") was used for dissecting "PA" and "MHV." The dissected regions are indicated by stippling. The nomenclature of frontal sections was based on the atlas of Karten and Hodos (1967): A, archistriatum; BO, bulbus olfactorius; CA, commissura anterior; CP, commissura posterior; CO, chiasma opticum; E, ectostriatum; FPL, fasciculus prosencephali lateralis; HA, hyperstriatum accessorium; HD, hyperstriatum dorsale; HV, hyperstriatum ventrale; LH, lamina hyperstriatica; LMD, lamina medullaris dorsalis; LPO, lobus parolfactorius; N, neostriatum; NC, neostriatum caudale; Ov, nucleus ovoidalis; PA, paleostriatum augmentatum; PP, paleostriatum primitivum; TO, tuberculum olfactorium; TSM, tractus septomesencephalicus; V, ventricle.

(From Rose and Csillag, 1985)

Experiment 2.1: Determination of whether all induced c-*fos* or c-*jun* was translated after different intensities of stress.

Transcription and translation of IEGs are under stringent control. Ideally there should be control at both stages. Since IEGs are intended to respond rapidly to stimuli, it therefore appears sensible to start the cascade as soon as possible after stimulation. When the cell has assimilated all the incoming information, where appropriate, attenuate and/or terminate their induction/expression. This would give the cell a relatively large time window to gain enough information to determine whether expression of IEGs is appropriate in response to a given stimuli.

The aim of this experiment was to determine whether control of c-*fos* and c-*jun* induction could occur at the level of translation. This was achieved by measuring the amount of c-*fos* and c-*jun* mRNA induced, and Fos and Jun proteins expressed after different types of stimulation. These results were expressed as a percentage of the induction, for either mRNAs or proteins, compared to their respective Quiet controls. If all the c-*fos* or c-*jun* mRNA induced was expressed as protein, then the percentage induction of mRNA compared to Quiet controls for chicks from the same test groups and region studied would be approximately the same as the percentage induction of its proteins compared to its Quiet control protein levels. Levels of induction were measured in the areas previously implicated in memory formation; both left and right IMHV and left and right LPO (Rose, 1991). I consider that inclusion of this experiment here is merited, even though the characterisation of Fos, Jun proteins are not mentioned until the next chapter.

METHOD

Animals and Training Protocol.

Ross 1 Chunky chicks (*Gallus domesticus*) of both sexes were hatched in a communal incubator on a 12-hours light/12-hours dark cycle at 38-40°C. On the evening of their hatching chicks were placed in pairs into 20x25x20 cm aluminium pens, illuminated with a 25-W red light. The birds were then left to equilibrate overnight with food and water. This period of time ensured c-*fos* had fallen to baseline levels.

The following morning chicks were assigned to groups and subjected to different forms of "stimulation". One set of chicks, Quiet controls, were left undisturbed for the duration of the experiment (Q). The other groups of chicks were trained according to the one-trial passive avoidance paradigm previously described by Lössner and Rose (1983). Briefly, chicks were pretrained by three 10 second presentations of a white bead (2.5 mm diameter). Chicks that pecked the white bead on at least 2 of the pretraining trials were then trained on a chrome bead (4mm diameter) dipped in either water (W) or the bitter tasting substance methylanthranilate (MeA). The remaining group was subjected to rough handling, by stroking and massaging the torso for 20 seconds and intraperitoneal injection of 200µl of 0.9% Saline [RS].

Induction of c-*fos* mRNA occurs within 5-10 minutes after stimulation and peak after 30 minutes, while protein expression reaches high levels 2 hours after stimulation (Greenberg and Ziff, 1984; Muller et al., 1984, Curran and Morgan, 1986). So, chicks from the four groups were killed at 30 minutes for their RNAs, or 2 hours for their proteins after stimulation. Each brain was rapidly removed and placed in the chick 'brain mould'. This enabled the brain to be held in the correct orientation for dissection into two large slices from which both LPO and IMHV could be quickly and easily removed (fig. 2.1). The

tissue was immediately frozen. To provide enough RNA for subsequent assays, the tissue from 6 chick brains was pooled.

The 30 minute samples were analysed for c-*fos* and c-*jun* mRNA (n=1 {6 chicks}) by Northern blot analysis. The 2 hour samples were assayed for Fos and Jun proteins by Western blot analysis (for protocol see chapter 3; n=2; ie. 4 chicks).

Statistical analysis of data

No statistical analysis was conducted owing to the low number of replicates.

RESULTS

The results demonstrate that induction of mRNA for c-*fos* and c-*jun* in response to W or RS 30 minutes after stimulation is similar to the induction of their protein in all regions studied. In contrast, in the group of animals trained on the MeA coated bead, there are high levels of c-*fos* mRNA induction compared to Quiet controls in the left LPO (700%), left IMHV (425%) and right IMHV (750%). This same degree of Fos proteins increase was not observed after Western blotting, where levels of induction were maintained around Quiet control levels (approximately 100% for all areas). There was no increase in c-*jun* mRNA (or its proteins) in response to the MeA coated bead. Results for the left and right IMHV are shown in fig. 2.3 and for the left and right LPO in fig. 2.4.

Figure 2.3 The effect of different forms of stress on the induction of c-*fos* or c-*jun* and their subsequent translation to Fos (p47 and p54) and Jun (p39 and p54) proteins in left and right IMHV. Chicks were subjected to three different treatments: training with a Water coated bead [W], training with a MeA coated bead [M] or rough handling [RS] -see text. Levels of c-*fos* and c-*jun* mRNA 30 mins, and Fos p47 and p54, and Jun p39 and p54 proteins 2h after the experiences were measured in left and right IMHV, and compared to levels expressed in Quiet controls (percent induction). No statistical analysis was performed.

Figure 2.3

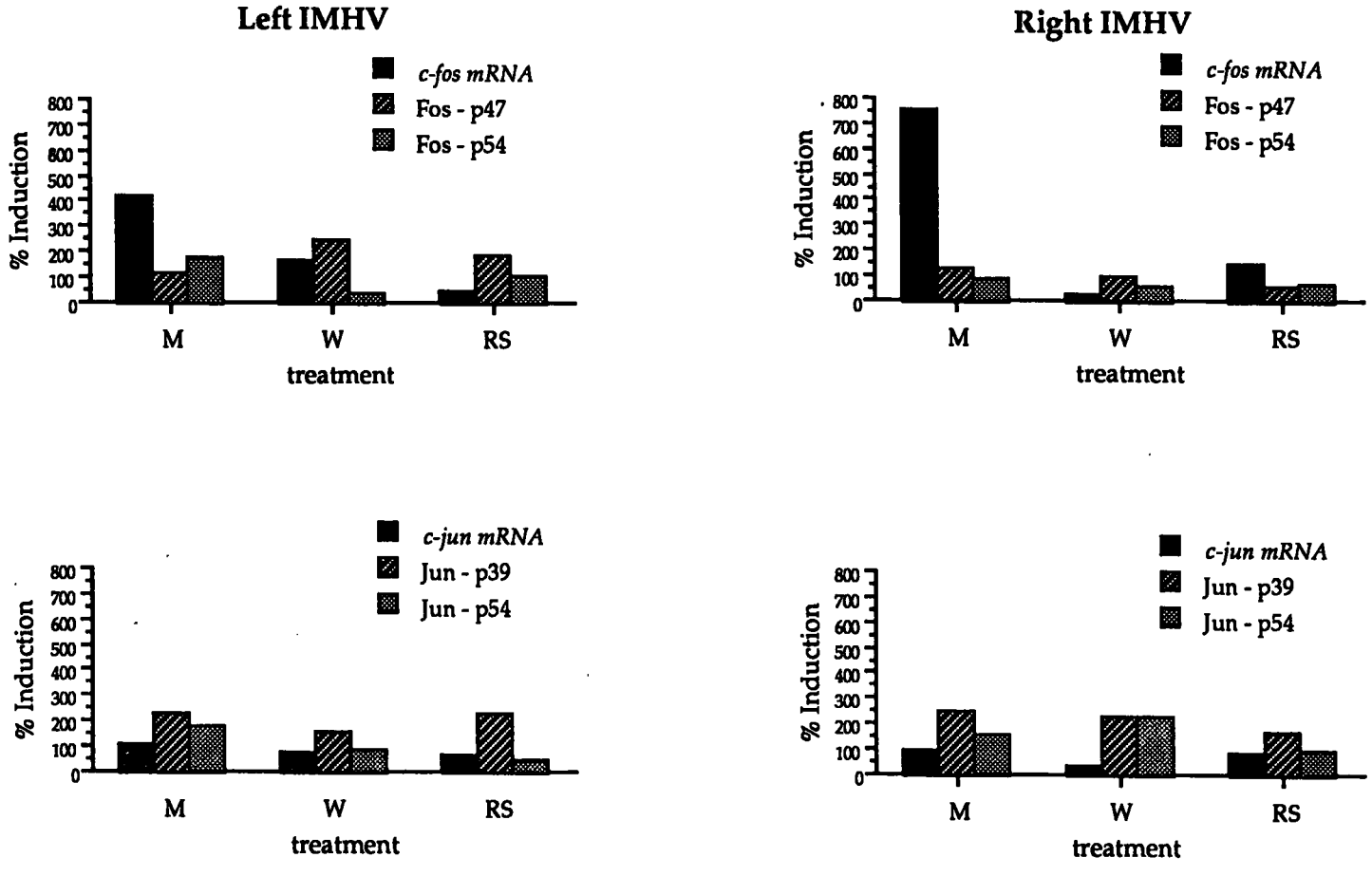
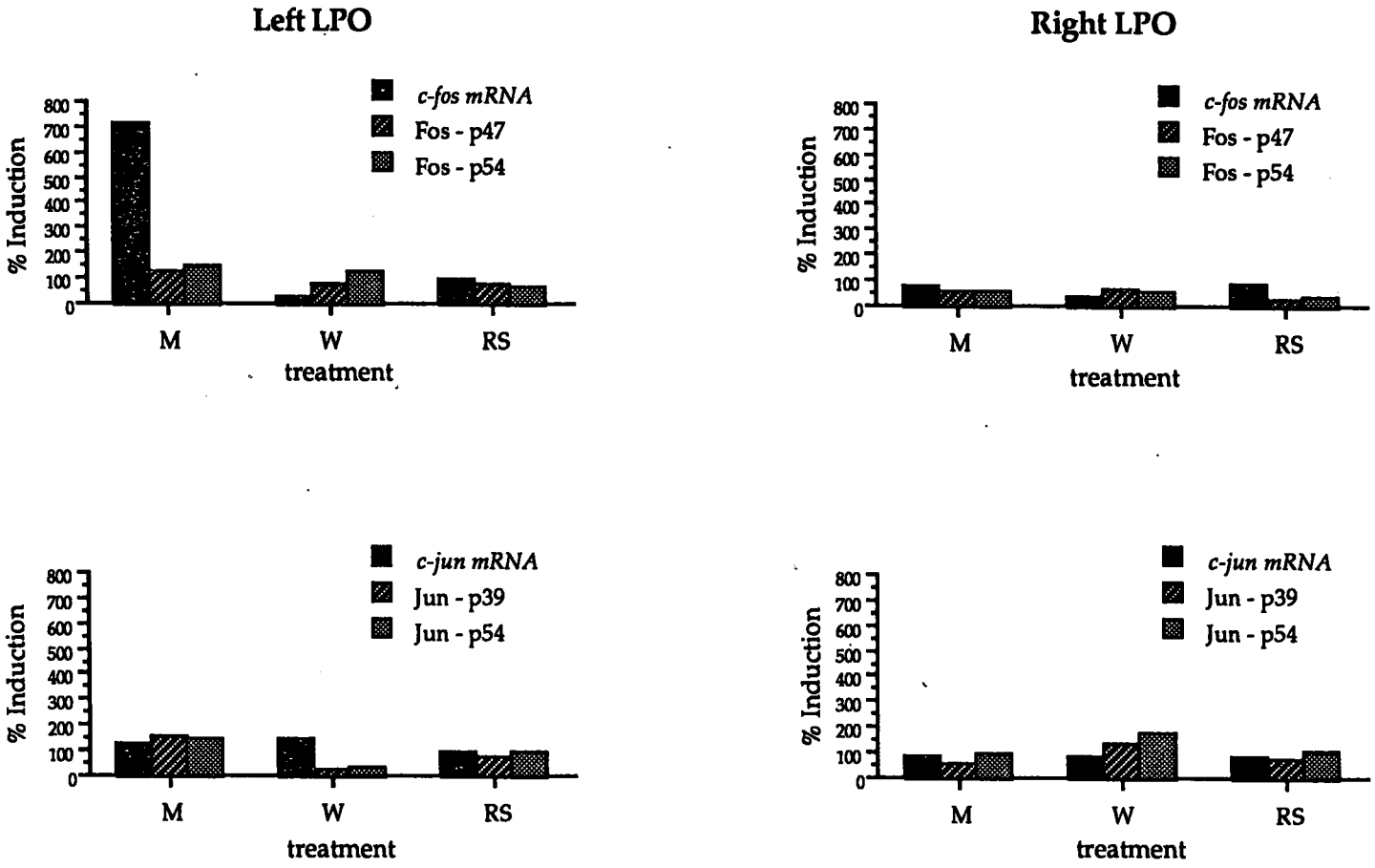

Left IMHV
c-fos mRNA
Fos - p47
Fos - p54
% Induction
800
700
600
500
400
300
200
100
0
M
W
RS
treatment
Right IMHV
c-fos mRNA
Fos - p47
Fos - p54
% Induction
M
W
RS
treatment
c-jun mRNA
Jun - p39
Jun - p54
% Induction
M
W
RS
treatment
c-jun mRNA
Jun - p39
Jun - p54
% Induction
M
W
RS
treatment

Figure 2.4 The effect of different forms of stress on the induction of c-*fos* or c-*jun* and their subsequent translation to Fos (p47 and p54) and Jun (p39 and p54) proteins in left and right LPO. Chicks were subjected to three different treatments: training with a Water coated bead [W], training with a MeA coated bead [M] or rough handling [RS] -see text. Levels of c-*fos* and c-*jun* mRNA 30 mins, and Fos p47 and p54, and Jun p39 and p54 proteins 2h after the experiences were measured in left and right LPO, and compared to levels expressed in Quiet controls (percent induction). No statistical analysis was performed.

Figure 2.4


Left LPO
Right LPO
c-fos mRNA
Fos - p47
Fos - p54
c-jun mRNA
Jun - p39
Jun - p54
% Induction
0
100
200
300
400
500
600
700
800
M
W
RS
treatment

DISCUSSION

The experiment (2.1) reported here indicates that transcription of c-*fos* was not a guarantee of translation after training in the chick. This was clearly evident in both left and right IMHV and left LPO where huge inductions of c-*fos* mRNA were observed but little or no increased expression of its proteins occurred. This was not the case for c-*jun* where transcription was closely related to translation. This experiment should be repeated using a larger sample in order to form any strong conclusions (i.e. show that transcription does not guarantee translation).

Fos protein has been shown to be induced in response to presentation of both MeA and Water beads 2 hours after training in the chick (Freeman and Rose, 1994). Since the object of experiment 2.1 was to determine if exposure to different stimuli caused IEG induction and expression, the birds were not tested for recall of their experience. Hence, a lack of protein expression after exposure to the MeA bead could have been due to the chicks not forming a memory for this experience.

Rough handling for 20 seconds followed by an intraperitoneal (i.p.) injection of saline did not induce c-*fos* or c-*jun* at all. However, Gubits et al., (1989) found that i.p. injection in rats resulted in the induction of c-*fos* mRNA. This induction could have been a consequence of their injection vehicle which was water and not 0.9% saline which was used here. Since Gubits et al failed to state the injection volume it is possible that the injection caused a temporary decrease in blood osmotic pressure, sufficient to induce c-*fos* (Gu et al., 1993). In addition, Gubits et al measured the level of c-*fos* mRNA in whole forebrains therefore not allowing exact identification of c-*fos* active areas. It could be that the analogous areas in rat, to chicks IMHV and LPO were not inducing c-*fos* in response to i.p. injections. In the paradigm employed here, i.p. injections of

saline appear not to induce c-*fos* or c-*jun*, thereby allowing drug administration via this route. This was particularly important in this case, because intracerebral injections are known to cause IEG induction (authors own observation), thus ruling out this route for drug administration. It was of interest that the stimulus I considered to be most stressful, rough handling, did not induce c-*fos* or c-*jun*. For this I can offer no sensible explanation. It only serves as a warning; do not be too ready to think that a chick will behave as you would to a stimulus.

Recently a method of measuring 2-DG uptake and c-*fos* induction on adjacent brain slices has been developed (Reimer, 1993). This method allows a direct comparison, on the same tissue, between the two methods. So one may determine whether different areas are exposed by the two methods, as originally thought. The initial findings appear to be quite promising. In the mouse, after acoustic stimulation, Reimer (1993) showed that in the inferior colliculus the c-*fos* mapping method gave a much more precise location of activity than 2-DG uptake did. Perhaps if a similar comparison between these methods were undertaken in the chick passive avoidance paradigm, one could discount non-specific c-*fos* induction.

In conclusion, whilst measurement of the time window of IEG induction is a much more sensitive metabolic marker than measuring expression of its proteins, one must also establish that transcription of these mRNA species occurs. If there is no transcription then there will be no activation of late genes by the transcription factors they form (e.g. AP-1).

CHAPTER 3

Characterization of anti-Fos and anti-Jun antibodies

INTRODUCTION

As stated in chapter 1, the measurement of IEG induction is a useful marker of neuronal activity. However, not all the c-*fos* mRNA induced in response to a bead coated in MeA was translated (see chapter 2). The measurement and identification of Fos or Jun proteins expressed in response to learning and memory formation would provide not only more convincing evidence for the involvement of IEGs in long-term memory formation but would also provide more information regarding this cascade. Since nothing is known regarding the species of Fos and Jun proteins expressed in the chick this chapter aims to identify them.

Synthesis of Fos protein is maximal 30 to 60 minutes after stimulation and degradation occurs with a half life of approximately 2 hours (Muller et al., 1984; Curran and Morgan, 1986). Once translated, Fos has the predicted molecular weight of 42 kDa. However, after sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE), it has been shown to run with an apparent molecular mass of between 55 and 62 kDa (Franza et al., 1987). This is because it undergoes rapid and extensive post-translational modification, with the majority of changes occurring within 15 minutes (Curran and Morgan, 1986; Muller et al., 1984). Polyclonal anti-Fos antibodies can also recognise Fos related antigens (Fras) (Sambucetti and Curran, 1986). Fras belong to the Fos gene family, and have the conserved Fos amino terminal but a slightly different carboxyl terminal. Since pheochromocytoma cells (PC12) behave as sympathetic neurones when stimulated by nerve growth factor (NGF), Franza

et al (1987) analysed the different types of Fos and Fras proteins expressed after stimulation by NGF. Five immunoreactive bands were observed that ran at 55-62 (Fos), 46 (Fra-2; Nishina et al., 1990), 40 (FosB), 35 (Fra-1; Cohen and Curran, 1988) and 30 kDa (Fra). The authors argued that the lower molecular weight bands were not breakdown products of the higher molecular weight bands. They reasoned that some bands were more evident under increasingly acidic conditions than others (eg. p40; a band that ran with a molecular mass of 40 kDa) and that the different kinetics of induction and half life of each band made it impossible for the lower molecular mass proteins to be derived from any higher mass bands.

In the caudate nucleus of the rat, Young et al (1991) found that after injecting the indirect dopaminergic agonist cocaine, a similar array of Fos immunoreactive bands which ran at 25, 35, 41, 44 and 55 to 69 kDa on SDS-PAGE gel were seen. Differences in the band positions between PC12 cells and rat brain could have arisen from different denaturing conditions during SDS-PAGE, different types of stimulation (and hence post-translational modification), and/or the different anti-Fos antibodies employed.

Jun-protein runs on SDS-PAGE at 39 kDa. Like Fos, it too is rapidly modified (Maki et al., 1987). Other species of the Jun family, which also differ by their amino terminal, such as JunB (Ryder et al., 1988) and JunD (Ryder et al., 1989), have been identified using polyclonal anti-Jun antibodies. Jun (and/or other members of the Jun family) can form homodimers or heterodimers with Fos (or Fras). On the other hand Fos (or Fras) can only form heterodimers. These dimers are the major constituent of the transcription factor, activator protein-1 (AP-1; Lee et al., 1987; Sonnerberg et al., 1989a). At least four other molecules are known to participate in the AP-1 complexes. Since AP-1s can contain a variety of molecules and, therefore, exist in many forms (species), it runs on SDS-PAGE gels with an apparent molecular mass of between 54-62 kDa.

Metrazole (MZT) has been shown to induce Fos and Jun proteins in the mammalian nervous system (Morgan et al., 1987; Saffen et al., 1988; Sonnenberg et al., 1989a-c; Zhu and Inturrisi, 1993). MTZ is believed to cause IEG induction by reducing GABA-ergic inhibition and causing membrane excitation, presumably via involvement of excitatory amino acids (EAA). This manifests itself in the animal experiencing seizures (Madeja et al., 1991; Zhu and Inturrisi, 1993). Fos levels have been shown to increase by a factor of approximately 20 one hour after MZT administration (50mg/Kg) (Curran and Morgan, 1987). Fos and Jun expression in the chick following passive avoidance training is believed to involve the activation of NMDA receptors, followed by a rise in intracellular calcium. Especially since administration of NMDA receptor antagonists not only block seziures but also effectively block immmediate-early gene activation (Sonnenberg et al., 1989a). The use of MTZ induced seizures serves as a good analogy by which to characterise Fos and Jun proteins in the chick brain.

This chapter describes the characterisation of Fos, Jun and their related proteins in the chick brain using Western blotting and localisation of these protein species by immunocytochemistry.

Experiment 3.1: Characterisation of chick brains Fos and Jun proteins by Western blotting.

METHODS

Training and injection of metrazole

Chicks were put into pens overnight and the following day injected with 2.5mg of MTZ in 0.2mls 0.9% saline (50mg/Kg) or left Quiet. In order to provide enough time for the translation of sufficient quantities of Fos and Jun

proteins, chicks were left to convulse for approximately 2 hours before being killed and assayed.

WESTERN BLOTTING

Quantification of the amount of Fos and Jun proteins expressed was carried out by Western blotting. Firstly, a known amount of protein homogenate was separated by SDS-PAGE; this was then blotted on to a nitrocellulose membrane. Specific bands of protein were then identified with the use of an anti-Fos (or anti-Jun) antibody which was visualised using an indirect peroxidase-anti IgG- diaminobenzene (DAB) or enhanced chemilluminescence (ECL) methods.

Antibodies

The antibody directed against Fos proteins was obtained from Prof. Peter Sharp (AFRC, Edinburgh). It was a polyclonal, raised in rabbit, against a 22 amino acid synthetic peptide corresponding to the c-terminal of chicken c-Fos. A Western blot of nuclear proteins from hypothalami of salt-loaded hens using this antibody revealed a single band at 47 kDa (Prof. P. Sharp per. comm.). The anti-Jun antibody was also rabbit polyclonal, purchased from Oncogene Sciences (PC07). It is known to detect Jun and AP-1 proteins. The Jun-antibody has not previously been characterised in chick brain (Oncogene Science, per. commun.). The secondary antibody was a horseradish conjugated to a goat anti-rabbit IgG (whole molecule; Sigma Chemicals plc.).

Gel preparation

For the identification of Fos and Jun proteins 5-15% gradient gels were made. The 5% acrylamide solution (5% acrylamide, 200mM Tris pH 8.8, 1% glycerol, 0.08% ammonium persulphate {APS}, 0.03%

tetramethylethylenediamine {TEMED}) and the 15% acrylamide solution (15% acrylamide, 200mM Tris pH 8.8, 10% glycerol, 0.03% APS, 0.03% TEMED) were placed in their appropriate compartments in the gradient making apparatus used to cast the gels. After approximately one hour the gels were set and a stacking gel added; 4% acrylamide, 1mM Tris pH 6.8, 0.075% APS, 0.025% TEMED. This was left to set for around 2 hours.

Sample preparation

Samples were prepared according to the purity of fraction desired.

(i) *homogenate*: A 10% (w/v) homogenate (PBS pH 7.2) was prepared.

(ii) *Crude nuclear prep:* A 10% (w/v) homogenate in buffer H (2mM Hepes, 1mM $MgCl_2$, 0.1M KCl, 0.32M sucrose) was prepared and centrifuged at 1000g max. The supernatent was collected for later use (i.e. testing for non-specific binding of the antibodies) and the pellet was homogenised in the same volume of buffer H as before. The sample was centrifuged as before and the final the pellet was resuspended in PBS (approximately 30% w/v).

(iii) *Clean nuclear prep:* The tissue was homogenised (20% w/v) in buffer H, then centrifuged at 1000g max for 5 minutes. The supernatant was collected for later use, and the pellet was homogenised in buffer H. The sample volume was brought up to 2.5ml with buffer H and the concentration of sucrose adjusted to 1.6M by the addition of 8ml gradient buffer (2mM Hepes pH 6.6, 1mM $MgCl_2$, 0.1M KCl, 2M sucrose). The homogenate was layered onto 8ml of gradient buffer in a centrifuge tube and dilution buffer (2mM Hepes, 1mM $MgCl_2$, 0.1 M KCl) was layered to the top of the tube. This was centrifuged for 105 minutes at 121,000g max. Three layers were revealed. The top layer contained cytosol, the middle layer contained membranes and the pellet contained nuclear material. The pellet was resuspended in 11ml of dilution

buffer and centrifuged at 121,000g for 30 minutes. Finally the pellet was resuspended in buffer H.

The protein content of each preparation was estimated using the Bradford method (Bradford, 1976; 5% sample in Bradford dye and the absorbance read at 595nm 10 minutes later). The protein concentration of each sample was adjusted to 2.5mg/ml with PBS. Fifty micrograms of protein was then mixed with an equal volume of sample buffer (0.25mM Tris pH 6.8, 4% SDS, 20% glycerol, 0.005% bromophenol blue,10% mercaptoethanol). This solution was boiled for 2-3 minutes to allow the proteins to unfold and become uniformly charged with SDS. The sample was then ready for loading onto polyacrylamide gels.

Separation and blotting of proteins on to nitrocellulose membranes

Once loaded on to the gel, the samples were run at 40V through the stacking gel and 60V in the resolving gel using running buffer (10% SDS, tank buffer (200mM glycine 25mM Tris-HCL, pH 8.3)). The protein bands were electroblotted onto a nitrocellulose membrane at 0°C and 20mA for 90 minutes in fix buffer (20% methanol, tank buffer).

Probing for proteins using the DAB system

The nitrocellulose membrane was blocked in BLOTTO (0.05% Tween 20, 5% marvel, TBS (50mM Tris, 0.145M NaCl, pH 7.6); Johnson et al., 1984) for 1 hour at room temperature, and then incubated over night at +4°C in 10ml of the primary antibody (rabbit anti-Fos) at 1:1000 (v/v) in BLOTTO. Three 10 minute washes in 50ml of BLOTTO prepared the membrane for the next incubation, using the secondary antibody, which was directed against the primary antibody (1:500 in 10ml of BLOTTO; v/v). This was left for 2-2.5 hours at room temperature. Again the membrane was washed twice in BLOTTO and once in

TBS/0.05% Tween 20, with each wash lasting 10 minutes. 50 ml of 0.05% DAB, 0.01% hydrogen peroxide was added to the blot. When bands could be visualised with sufficient intensity, the reaction was quenched by washing with copious quantities of water.

Probing for proteins using the ECL system.

After blotting, the membrane was blocked in 50mls of modified BLOTTO (10% marvel, 0.1M PBS, pH 7.4) overnight at +4°C. The blot was washed in 0.01% triton-PBS for 5 minutes, prior to incubation, with the primary antibody (anti-Fos at 1:1,000 (v:v) in 1% marvel, PBS) for 2 hours at room temperature, whilst rocking. The membrane was then thoroughly washed for 20 minutes in each of the following; (i) 0.05% triton, PBS (ii) 0.5M NaCl, 0.05% triton-PBS (iii) 0.05% triton-PBS. The secondary antibody incubation, at 1:500 in 1% marvel-PBS, was left at room temperature for 1 hour. Again the membrane was thoroughly washed. The membrane was now ready to be probed with the Amersham ECL kit solution according to the manufacturers' protocol. That is, the membrane was slowly agitated with 0.125ml/cm^2 (final volume) of freshly made detection reagent for exactly 60 seconds. Excess detection reagent was drained off and the membrane wrapped in SaranWrap. The membrane was then apposed to autoradiography film (Kodak) for between 15 seconds and 5 minutes, until the bands detected after development (5 minutes developer, 10 minutes fix) were of satisfactory intensity. The membranes were then washed ready for stripping and reprobing. The resultant autoradiogram immunoreactive bands were quantified using a Joyce Loebe chromoscan. Film linearity of response was monitored by apposing for different time periods. For each film the intensity of bands derived from stimulated birds were compared to the intensity of Quiet (unstimulated birds) bands. These results were expressed as percentage induction. As long as the films response was linear (ie.

not over or under exposed) the percent induction for each test band should be approximately the same for each film.

Stripping and reprobing of membranes

After ECL treatment, the membrane was washed twice for 10 minutes in TBS-T (0.1% Tween 20, 0.1M TBS, pH 7.4.) All antibodies were then stripped off the membrane by immersing in stripping buffer (2% SDS, 100mM mercaptoethanol, 62.5mM Tris-HCL, pH 6.8) for 30 minutes at +70°C. The membrane was then washed as before in TBS-T.

Blocking and probing for immunoreactive Jun bands was conducted exactly as stated above except that the primary antibody was anti-Jun (not anti-Fos) used at 1:500 (v/v). This method yielded two autoradiograms for each gel, one Fos and one Jun. This facilitated a direct comparison between the autoradiograms.

RESULTS

Many bands were observed when the homogenate was probed for Fos immunoreactivity; bands were detected by either the DAB (fig. 3.5) or ECL method (figs. 3.1 and 3.5). The crude and clean nuclear preparations showed 2 protein bands with Fos immunoreactivity, which ran at 47 kDa and 54 kDa (AP-1 binding protein). The antibody to Jun revealed two immunoreactive bands at 39 kDa and 54 kDa (AP-1 binding protein). The two Fos bands were easily seen using the ECL system (fig. 3.4. and 3.5) but were very faint at the concentration of sample loaded (50 µg) using the DAB method (fig. 3.2). Both methods showed that contaminants such as membranes and cytosol (figs 3.3 and 3.6) were responsible for the non-specific binding which was evident in the homogenate samples (figs. 3.1 and 3.3).

such as membranes and cytosol (figs 3.3 and 3.6) were responsible for the non-specific binding which was evident in the homogenate samples (figs. 3.1 and 3.3).

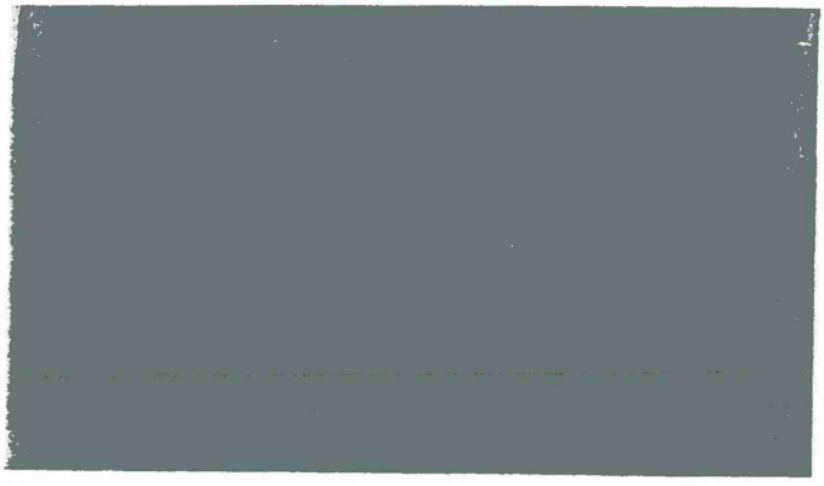


Figure 3.1. Membrane after separation of crude homogenate sample and probing for Fos immunoreactivity using the DAB system of visualisation.

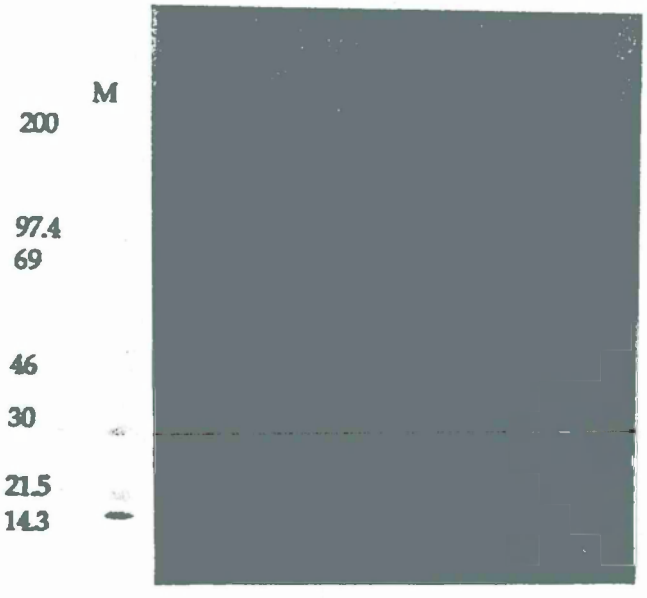


Figure 3.2. Autoradiogram after separation of a crude homogenate sample and probing for Fos immunoreactivity using the ECL system of visualisation. Exposure time, 1 minute.

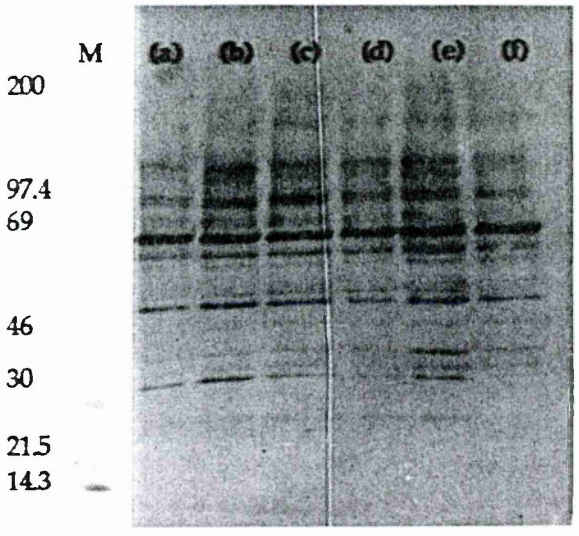
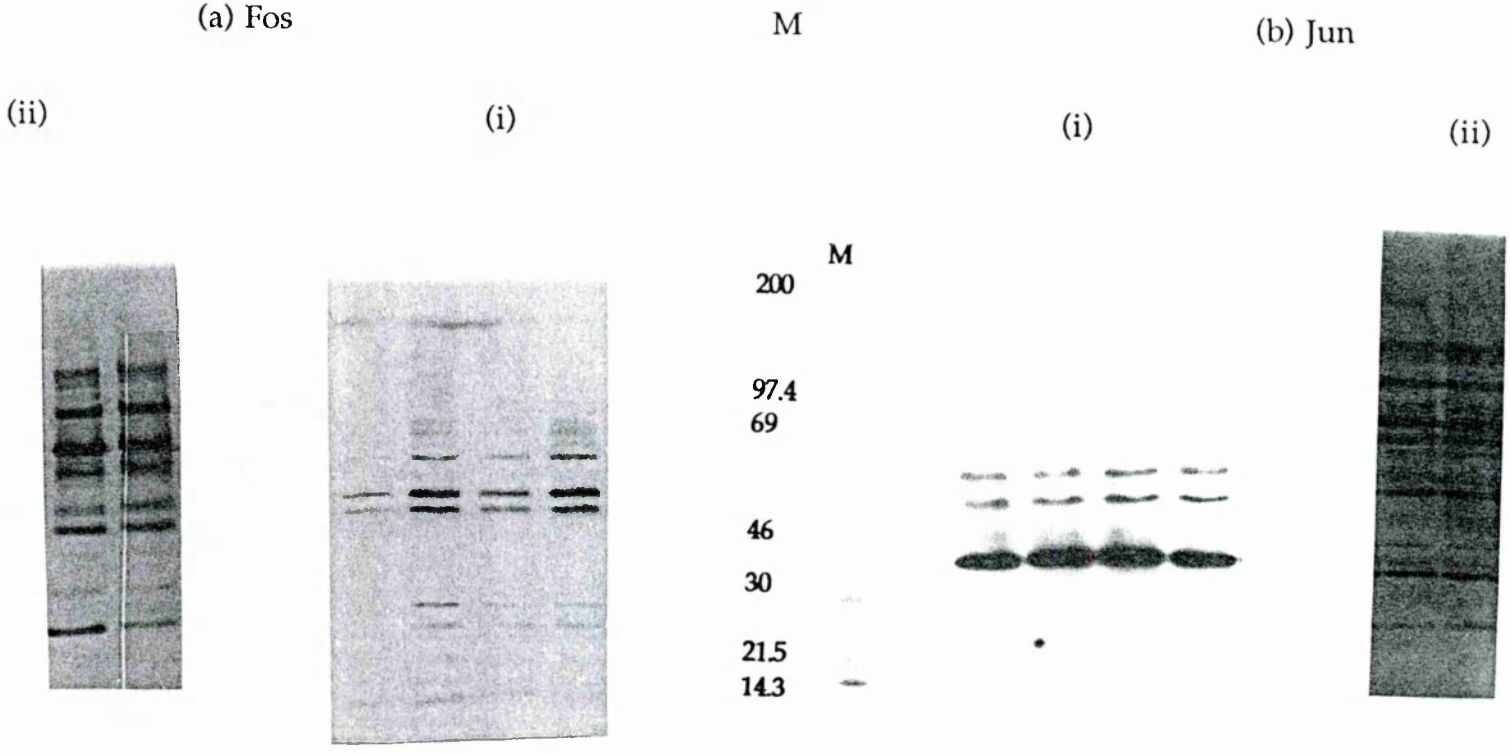


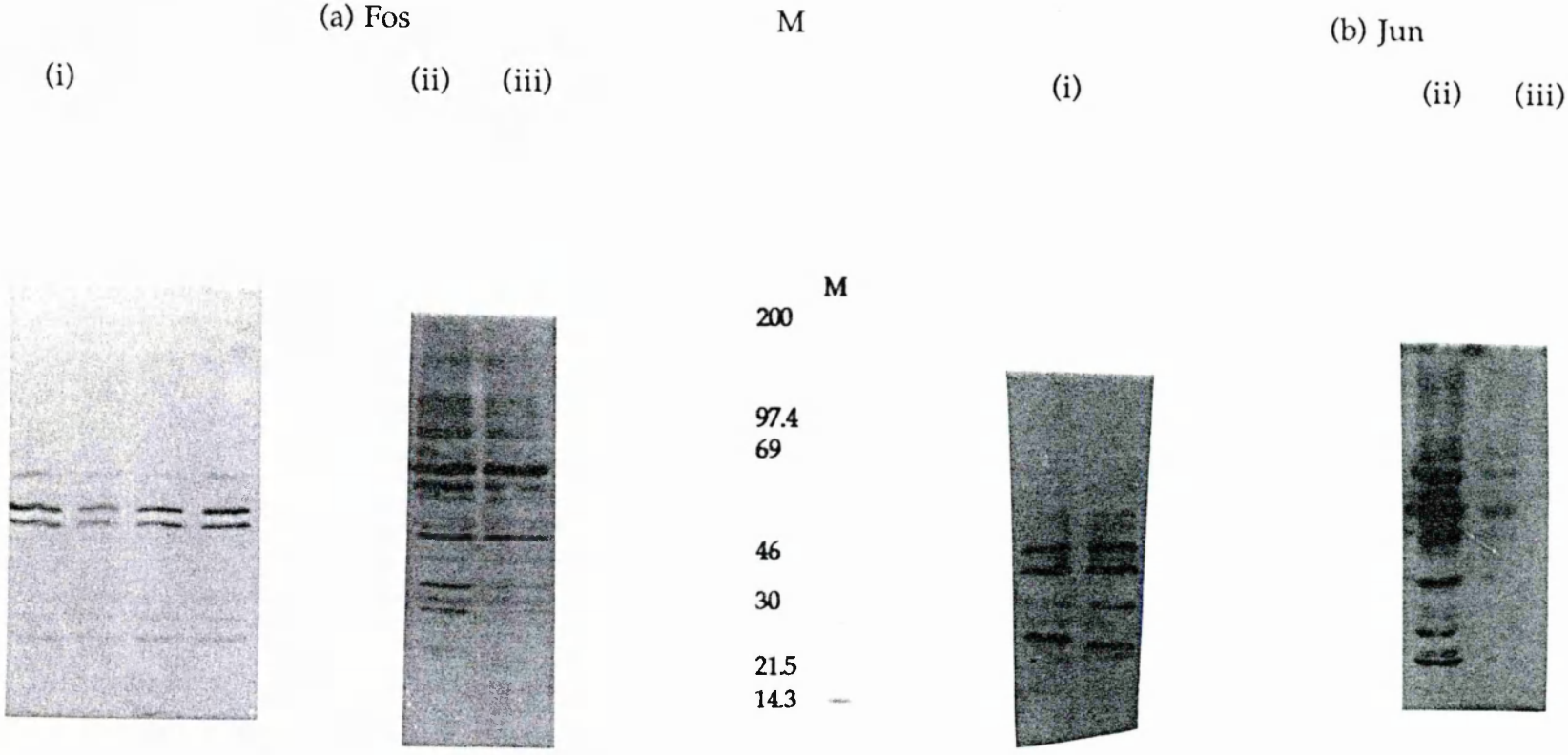
Figure 3.3. Autoradiogram after separation of a crude homogenate sample and probing for Jun immunoreactivity using the ECL system of visualisation. Exposure time 1 minute.

Figure 3.4.



Autoradiograms after separation of samples derived from a crude nuclear prep (i) nuclear prep and (ii) its supernatant, and probing for (a) Fos or (b) Jun -immunoreactivity using the ECL system of visualisation. Lane M contains molecular weight markers.

Figure 3.5.



Autoradiogram after separation of a samples derived from a clean nuclear prep (i) nuclear prep, (ii) membranes and (iii) cytosol, after probing for (a) Fos or (b) Jun -immunoreactivity using the ECL system of visualisation.

Experiment 3.2: Location of Fos and Jun proteins within the neurone by immunocytochemistry.

METHOD

Training and injection of metrazole

As in experiment 3.1, chicks were put into pens over night. The following day chicks were either injected with MTZ or left Quiet. Birds were left to convulse for approximately 2 hours. The chicks were then ready for perfusion.

Tissue preparation

Chicks were anaesthetized with sagatol (phenobarbitone) and perfused through the heart with saline followed by Zamboni's fixative (15% picric acid , 4% paraformaldehyde, 0.1M PBS; Zamboni and DeMartino, 1985). The brains were then removed and left overnight in Zamboni's fixative before washing in PBS (Zamboni and DeMartino, 1985).

Brains were rapidly frozen in an iso-pentane/solid CO_2 bath, then mounted on a chuck for cryostating. Slices, 20μm thick, were cut from areas of the brain which contained the LPO and IMHV. Sections were collected every 200μm from each area. This allowed a mean density of positive stained nuclei per region per chick to be calculated. Also, 3 successive sections were collected for anti-Fos probing, anti-Jun probing and histological staining. Sections were mounted on chrome-alum-gelatine coated slides. The slides were stored at -70°C for up to 1 month prior to immunocytochemistry.

IMMUNOCYTOCHEMISTRY

Sections were left to warm up to room temperature and then washed for three 10 minute sessions in PBS to remove excess Zamboni's fixative. Quenching of endogenous peroxidase activity was achieved by incubating sections in PBS containing 1% hydrogen peroxide for 30 minutes. After washing with PBS, non-specific binding was blocked by incubating sections for 30 minute in PBS containing 10% normal goat serum (NGS). The slides were then left overnight at room temperature in a solution of anti-Fos (or anti-Jun) antibody (1:9,000; (v/v) in 1% NGS/PBS). The following day, after stringent washes with PBS the slices were incubated overnight at +4°C with a peroxidase labelled anti-rabbit secondary antibody at 1:100 (v/v) in 1% NGS/PBS.

All secondary antibody was removed by washing twice for 20 minutes in PBS and 20 minutes in 50mM TBS. The peroxidase labelled secondary antibody was probed for by a short 5 minute incubation with 0.03% H_2O_2/TBS followed by an additional incubation with the same solution except that this time it contained 0.01% DAB. The reaction was terminated with copious quantities of TBS. Cover slips were placed over fixed sections and blue/black staining of DAB was visualised using a light microscope.

RESULTS

Both anti -Fos (fig. 3.6) and -Jun (fig 3.7) immunocytochemistry showed that labelling was confined to the nucleus with little/no staining in the cytosol. In the Quiet control group there were very few Fos- or Jun- positive nuclei (figs 3.8. and 3.9) indicating that the expression seen in the treated birds was due to MTZ injection. A further control group was included which showed little/no non-specific staining if incubation with primary antibodies were omitted.

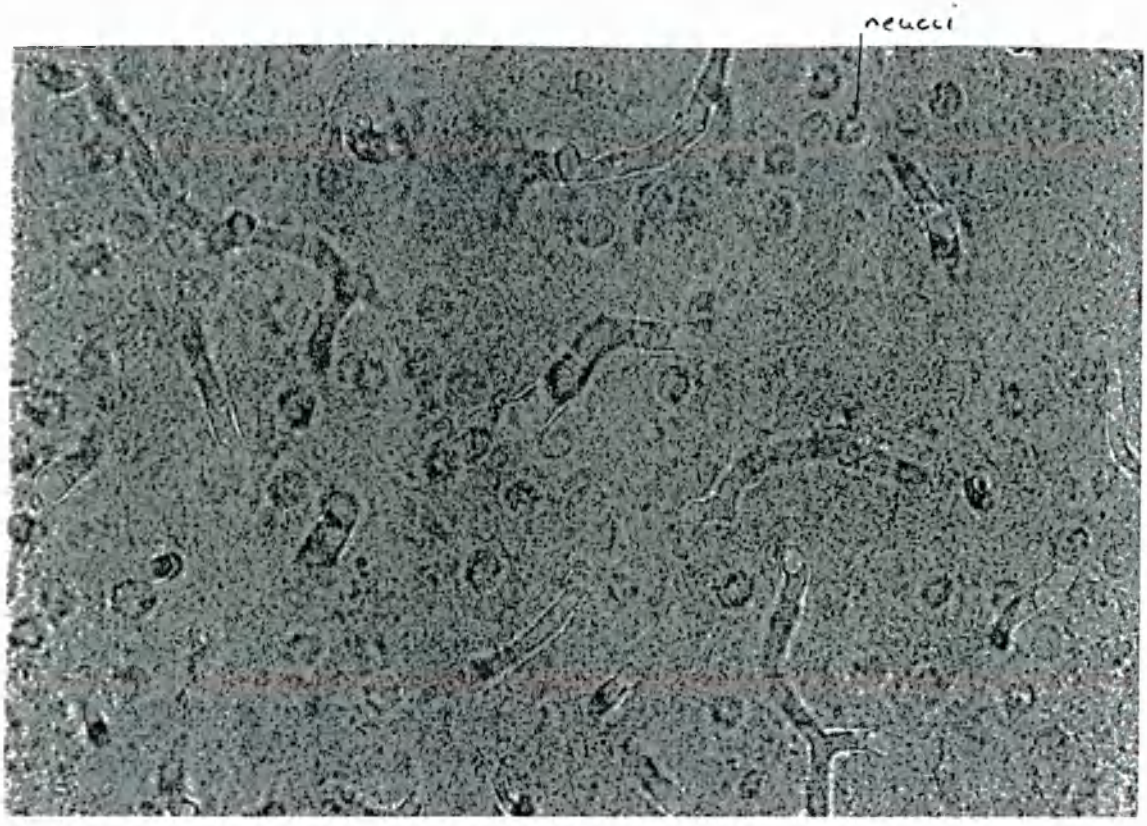


Figure 3.6. Coronal section of chick brain 2h after MTZ treatment followed by anti-Fos immunocytochemistry .

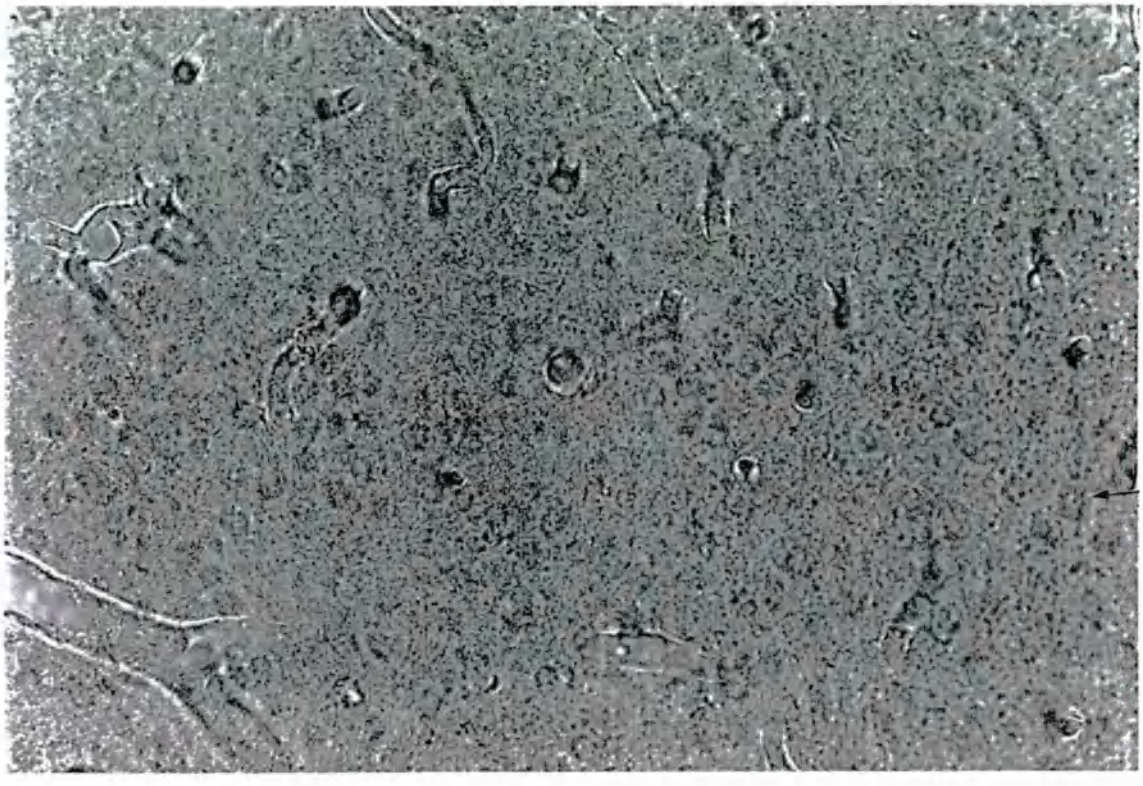


Figure 3.7. Coronal section of chick brain 2h after MTZ treatment followed by anti-Jun immunocytochemistry.

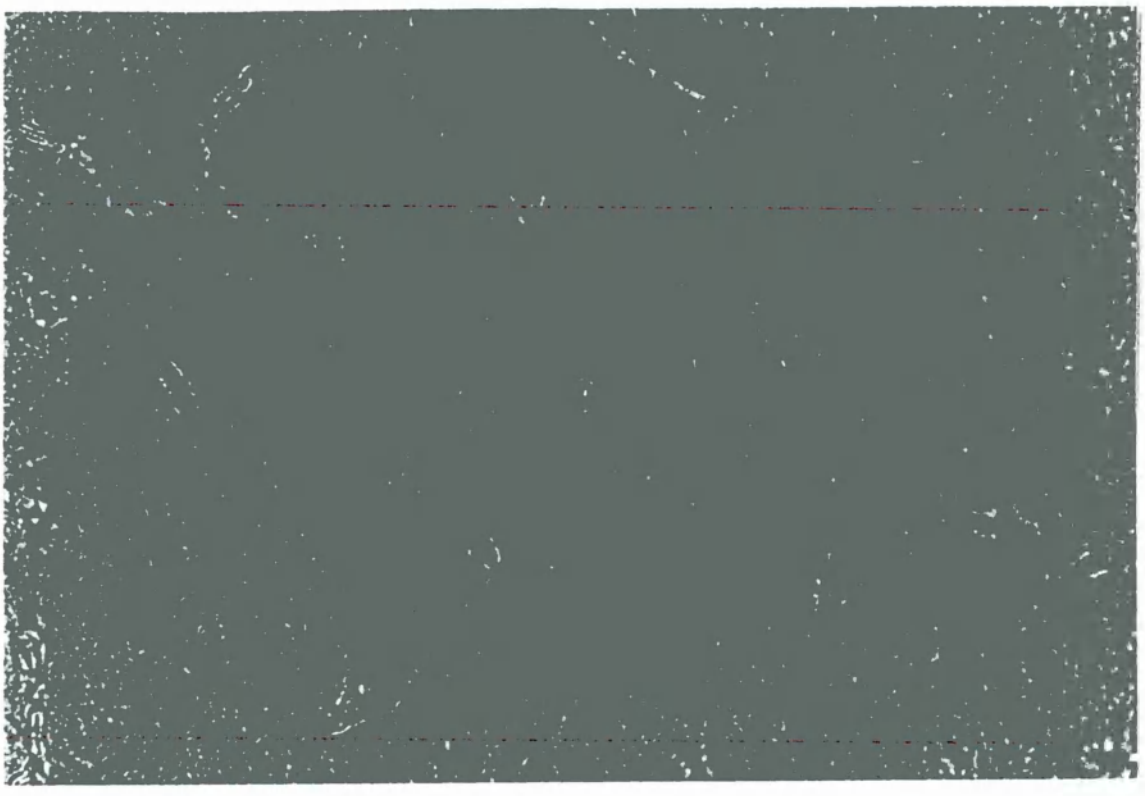


Figure 3.8. Coronal section of a Quiet control chick brain after anti-Fos immunocytochemistry.

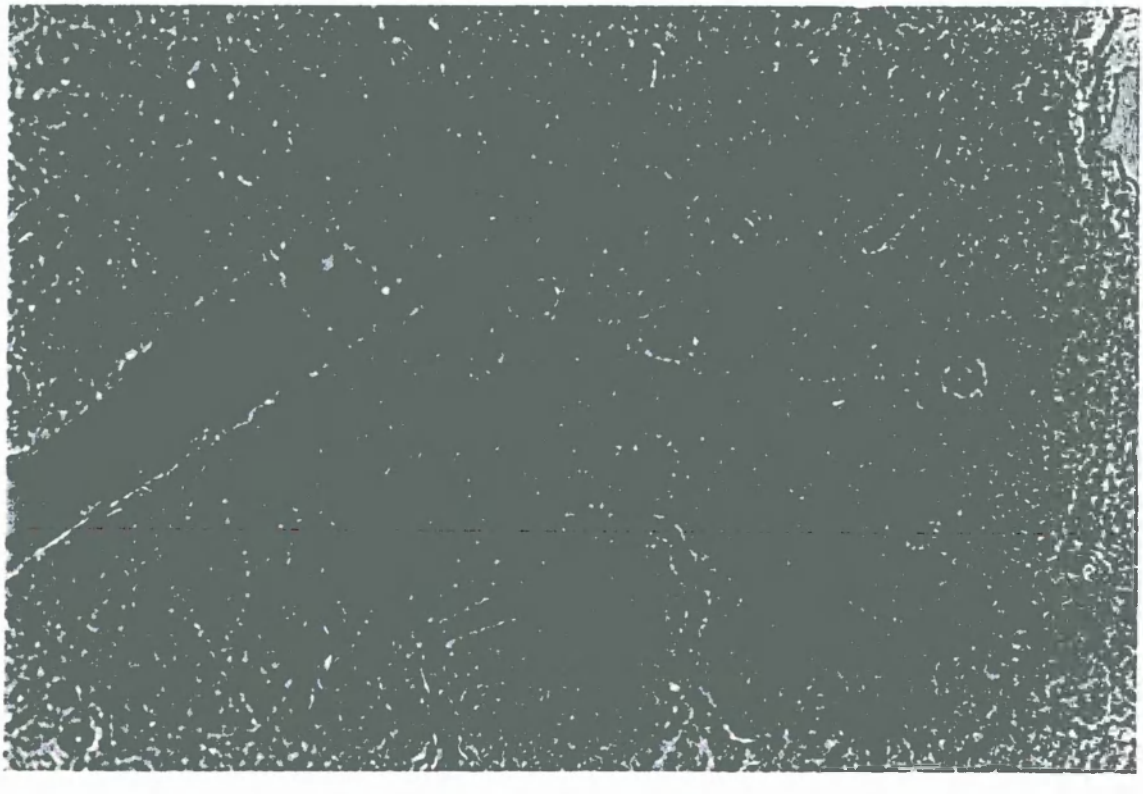


Figure 3.9. Coronal section of a Quiet control chick brain after anti-Jun immunocytochemistry.

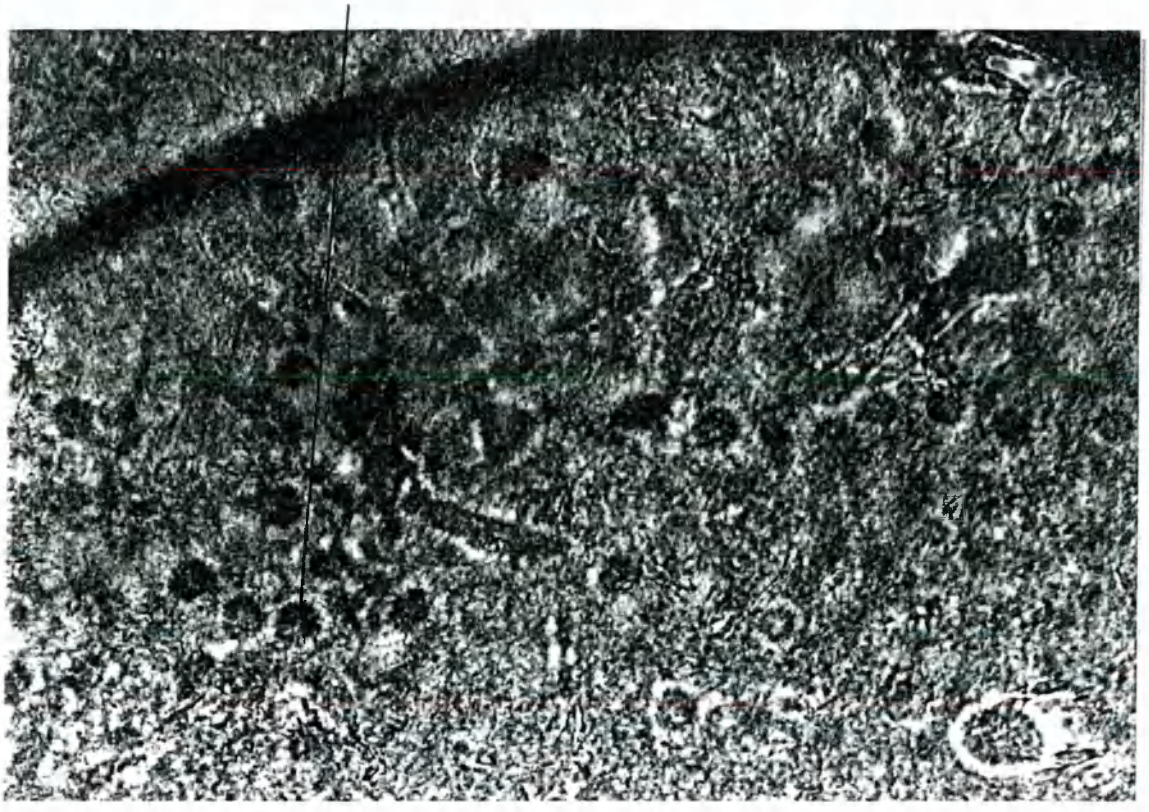


Figure 3.10. Coronal section of chick brain 2 hours after MZT treatment at a magnification of X60 showing Fos/DAB-stained nuclei.

DISCUSSION

Many bands were observed from samples of pure homogenate by both the ECL and DAB methods of visualisation. Control blots which included no primary antibody demonstrated that this was due to the non-specific binding of both the anti- Jun and Fos antibodies. This was probably due to the large quantity of sample required for any band detection. Western blotting using the ECL method of visualisation confirmed the manufacturer's claim that it was far more sensitive than the DAB method of band identification. Consequently, less sample was required for good separation and identification, so that proportionally less contaminates were loaded onto the gel. As a result no non-specific bands were seen in either the crude or clean nuclear preparation.

Following seizures induced by MTZ, 2 protein bands with Fos immunoreactivity at 47 kDa and 54 kDa were induced. Based on P.J. Sharps

observations, I believe the 47 kDa to be Fos. The 54 kDa band that was recognised by both antibodies could be an AP-1 protein. However, only further investigations into the structure of this protein band will elucidate this. The 39 kDa Jun immunoreactive band could be Jun protein, since Jun is known to have this molecular mass (Maki et al, 1987). Thus, if MTZ seizures induces these Fos and Jun proteins through EAA activation, and long-term memory formation in the chick is believed to involve NMDA activation followed by IEG expression, then if any induction is going to be seen in response to passive avoidance training, the expression of these species of Fos and Jun proteins will be evident. In addition, since immunocytochemistry showed that both Fos and Jun proteins were isolated to the nucleus, in memory formation, any subsequent IEG expression will be confined to the nucleus.

CHAPTER 4

Training-induced Fos and Jun proteins; a time-course study

INTRODUCTION

Induction of Fos and Jun proteins following IEG activation in the brain has been demonstrated in response to many stimuli. In the rat, noxious stimulation (Bullitt, 1990; Hunt et al, 1987), focal cerebral ischemia (An et al, 1993; Uemura et al, 1991), induced effects of water deprivation (Gu et al, 1993), chemical seizures (Morgan et al 1987), electrical stimulation (Dragunow and Robertson, 1987), kindling and induction of LTP (Dragunow et al, 1989a; 1989b) lead to Fos and Jun expression. Fos and Jun proteins have also been induced in the mouse in response to light stimulation and brain injury (Nir and Agarwal, 1993; Dragunow and Robertson, 1988).

The induction of IEGs are considered to be important in providing the "building blocks", i.e. proteins and glycoproteins for this synaptic remodelling. In the mouse, a learning related induction of Fos and Jun after an appetitive bar-pressing task has been observed (Heurteaux et al, 1993). In the avian brain, Szekely et al (1992) demonstrated that following a spatial memory task in marsh tits, there was an induction of Fos in both forebrain hemispheres in the HV, LPO and ARCHI. Using the chick passive avoidance training paradigm, Anokhin et al (1991) showed that c-*fos* mRNA was induced in both right and left LPO and IMHV. In another experiment using an associative learning task, the pebble floor, Anokhin and Rose (1990) again described the induction of c-*fos* and c-*jun* mRNA in these same regions in the chick. Imprinting in the chick also causes induction of c-*fos* mRNA in LPO and IMHV (Abramova et al., 1992).

However, Hunt (1992), Kiessling (1993) and myself in the preceding chapter showed that even though c-*fos* and c-*jun* mRNA can be induced their proteins were not expressed. If IEGs are not translated then they cannot act as transcription factors in the control of late gene activation. In addition, the study of c-*fos* or c-*jun* mRNA induction gives no indication of post-translational modification and hence the species of transcription factors formed such as AP-1. Knowledge of the species of transcription factor and the promoter/enhancer sequences of late genes can help to elucidate which genes are activated (or repressed), so allowing the identification of proteins which are expressed and utilised in response to long-term-memory formation and retention.

The aim of these experiments were to determine, using the antibodies characterised in chapter 3, whether Fos and Jun proteins are induced in either or both left and right IMHV and LPO at various times after training.

Experiment 4.1: Fluctuations throughout a 10hr period in the quantity of expressed Fos-proteins.

The aim of this chapter is to determine whether Fos or Jun proteins are expressed in response to passive avoidance learning. The simplest method of quantifying this induction is to express it as a percentage of basal levels found in Quiet control birds killed at the same time as test birds. Fluctuations in the expression of Fos (or Jun) could be quite large throughout a day, due to chicks learning from cues other than training (eg. smell, noise), and/or fluctuations in hormonal levels as governed by circadian cycles or stress. Thus, if *c-fos* induction is an all or nothing relationship as Morgan and Curran (1991) seem to think, then the amount of Fos needing to be induced due to learning could differ throughout the day. If the level of Fos proteins is high at a particular time, this could bias the results to show that very little Fos had been induced due to the learning experience. Whereas, when the level of Fos is low a bias to large induction of Fos would be seen. Hence it would be useful to determine whether Fos levels do fluctuate greatly through the day. This would enable one to determine the optimum time to kill Quiet control birds, at the beginning of the experiment or when test birds are killed.

METHOD

Tissue collection

As usual birds were left to equilibrate over-night, ensuring that Fos levels had fallen to low concentrations. Absolute Quiet control chicks were killed at 09.00hrs. The remaining chicks were decapitated, 1, 2, 3, 4, 5, 6, 7, 8 and 10 hours subsequently. Both left and right IMHV and LPO were collected for Fos quantification by Western blotting (for method see chapter 3).

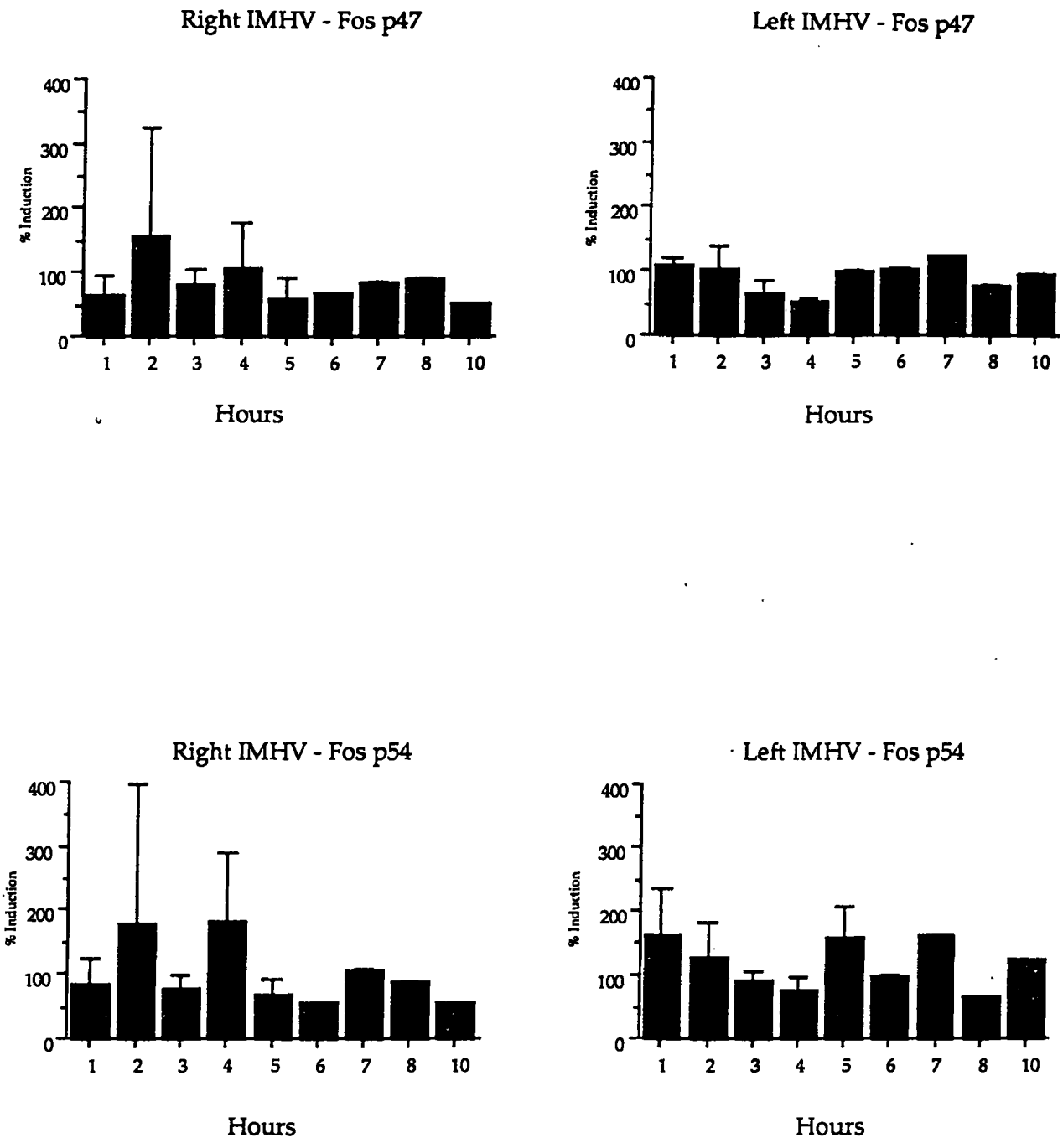
Treatment of data

Results for each time point were expressed as the percentage level of Fos expression compared to Absolute Quiet controls, for the individual regions studied. Since the numbers of chicks used at each time point were low (n = 1-3) no statistical analysis was conducted.

RESULTS

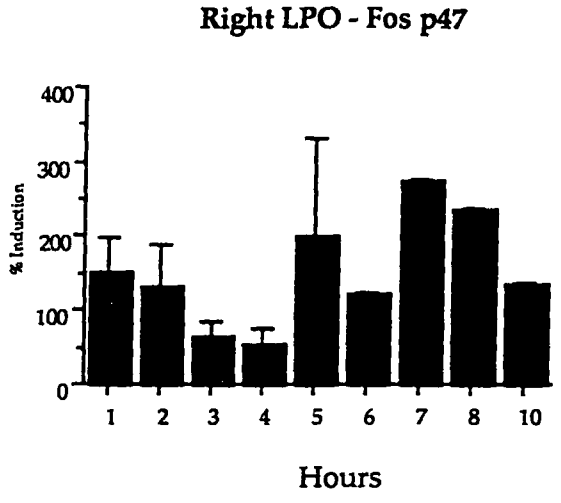
The results demonstrate that in the left IMHV there was little fluctuation in the amount of Fos (p47 and p54; figure 4.1). There was also little fluctuation in Fos p47 and p54 levels in the right IMHV, except for the 2 and 4 hour time points where there was a large variation in values. Both LPOs exhibit large fluctuations in Fos p47 and p54 expression during the 10 hours period of the experiment (figure 4.3). These large variations were presumably exaggerated by a small sample size.

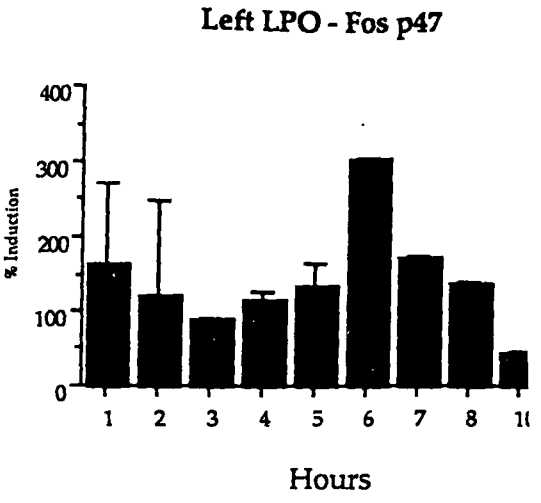
Figure 4.1

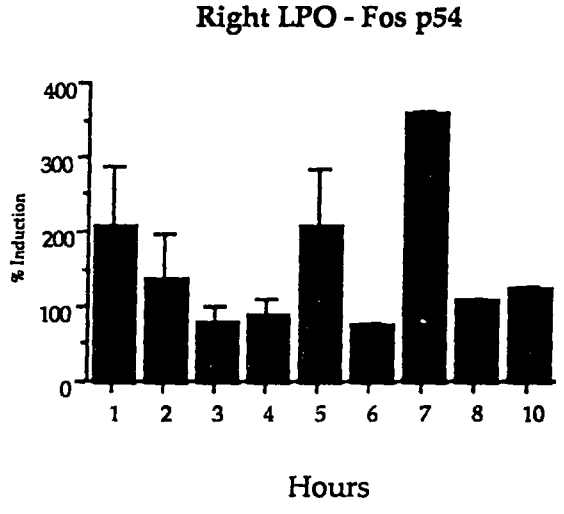


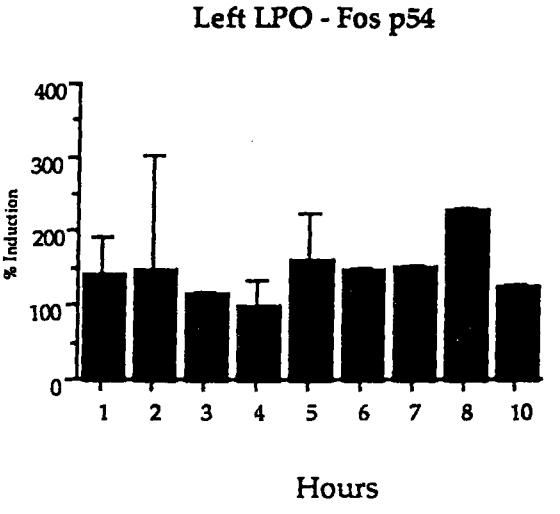
Fluctuations in Quiet levels of Fos p47 and p54 protein expression in a 10 hour period. The level of Fos proteins in the IMHV were compared between Absolute Quiet birds and birds left Quiet and killed at different times from 09.00h to 19.00h.

Figure 4.2









Fluctuations in Quiet levels of Fos p47 and p54 protein expression in a 10 hour period. The level of Fos proteins in the LPO were compared between Absolute Quiet birds and birds left Quiet and killed at different times from 09.00h to 19.00h.

DISCUSSION

Since both the left and right IMHVs showed little fluctuation and both LPOs showed large fluctuation in Fos levels, the most practical course of action to employ would be to kill Quiet control birds at the start of the experimental day and Quiet birds killed at the same time as test birds. A comparison of Fos levels from both sets of Quiet birds would act as an indicator of excessively high levels of expressed Fos. However, the results show that there are times when the Absolute Quiet birds express the largest level of Fos (eg right IMHV and right LPO). Owing to the large fluctuations of Fos expression which appears to be day (batch) -specific and the impracticability of collecting so many Quiet birds, I have decided to adopt a protocol of collecting tissue from Quiet birds at the same time as test birds. I believe this Quiet control to be better than an Absolute Quiet control because one at least gets a better idea of "extra-learning" stimulation up to the time of killing than using the former control.

Experiment 4.2: Time-course of Fos and Jun expression after passive avoidance training in the chick.

The aim of this experiment was to determine whether passive avoidance training induced the expression of Fos and Jun proteins in LPO or IMHV at various times post-training. Since many forms of stimulation are known to induce IEG expression, a control group trained on a Water coated bead was also included. Any increase in IEG expression derived from the act of offering and pecking at the bead could therefore be dissociated from long-term memory formation for avoidance.

METHODS

Animals and Training Protocol.

Ross 1 Chunky chicks (*Gallus domesticus*) of both sexes were hatched in a communal incubator on a 12-hours light/12-hours dark cycle at 38-40°C. On the evening of their hatching chicks were placed in pairs into 20x25x20 cm aluminium pens, illuminated with a 25-W red light. The birds were then left to equilibrate overnight with food and water. This period of time ensured c-*fos* had fallen to baseline levels.

At 09.00h the following morning chicks were allocated to groups. One set of chicks, Quiet controls, were left undisturbed for the duration of the experiment. The other groups of chicks were trained according to the one-trial passive avoidance paradigm previously described by Lössner and Rose (1983). Briefly, chicks were pretrained by three 10 second presentations of a white bead (2.5 mm diameter). Chicks that pecked the white bead on at least 2 of the pretraining trials were then trained on a chrome bead (4mm diameter) dipped in either the bitter tasting substance methylanthranilate (MeA) or water (W). After training birds were tested for their recall of the training experience by presentation of a dry chrome bead. MeA birds that pecked the chrome bead on test were amnesic; those that avoided the bead showed recall of the task. In a pilot study, chicks were tested for recall at 1, 2, 3, 4, 5, 6, 7, 8 or 10 hours after training. Birds trained on MeA which avoided (recall) and Water birds that pecked the test bead, plus Quiet controls for that time point, were decapitated and both LPO and IMHV collected. A crude nuclear preparation for each sample (2 birds) was assayed for Fos and Jun proteins by Western blotting as previously described (chapter 3). Analysis of the pilot study results indicated the requirement for collection of additional samples for only the 1 - 4 hour time points.

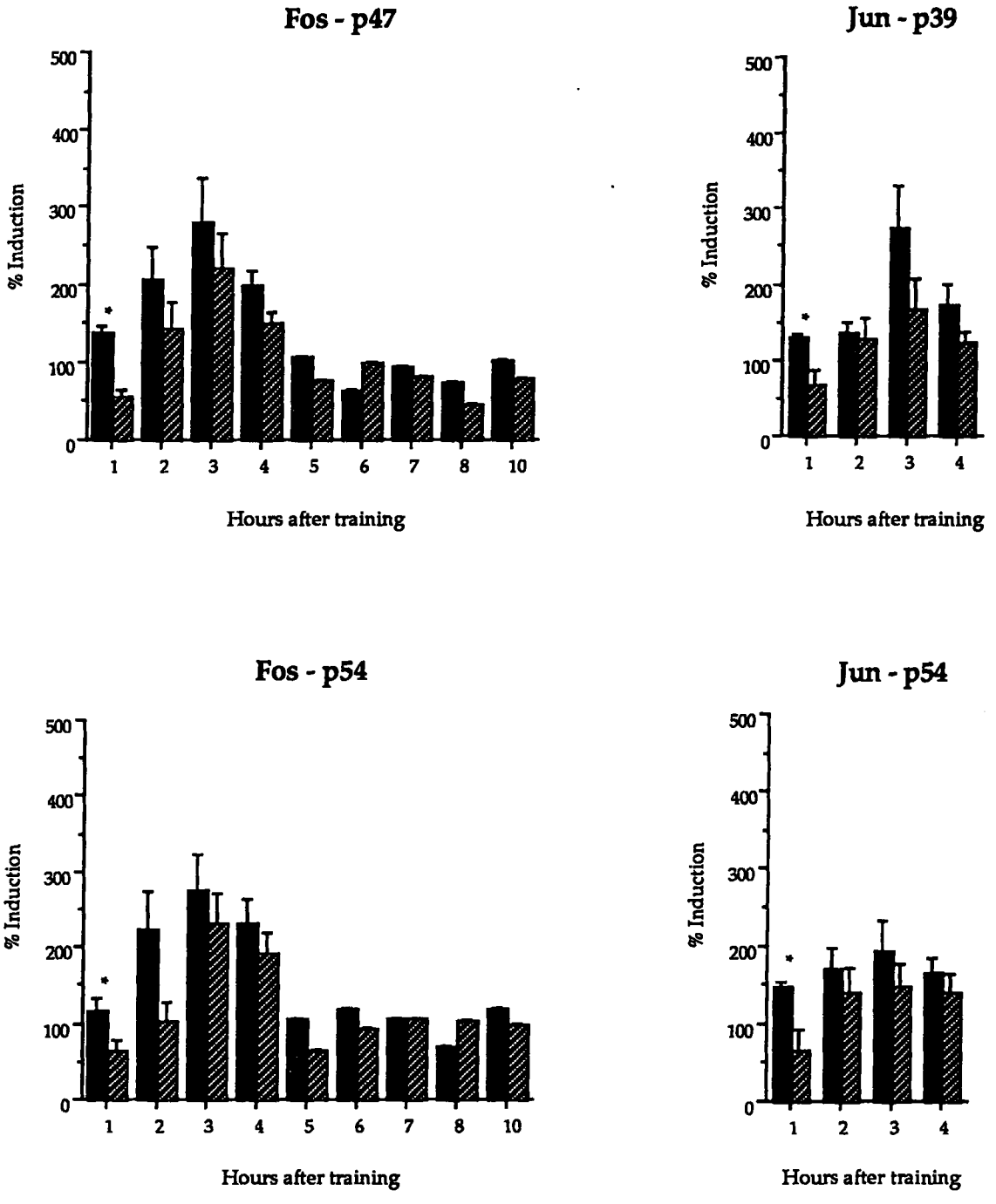
Statistical treatment of data

Results were expressed as the percentage induction for each protein peak compared to basal levels (Quiet chicks) of samples run on the same gel. Statistical analysis was only conducted on samples collected between 1-4 hour post-training. Where appropriate, comparisons between MeA and Water birds for each protein species, time point and region, were performed using a Mann-Whitney U test.

RESULTS

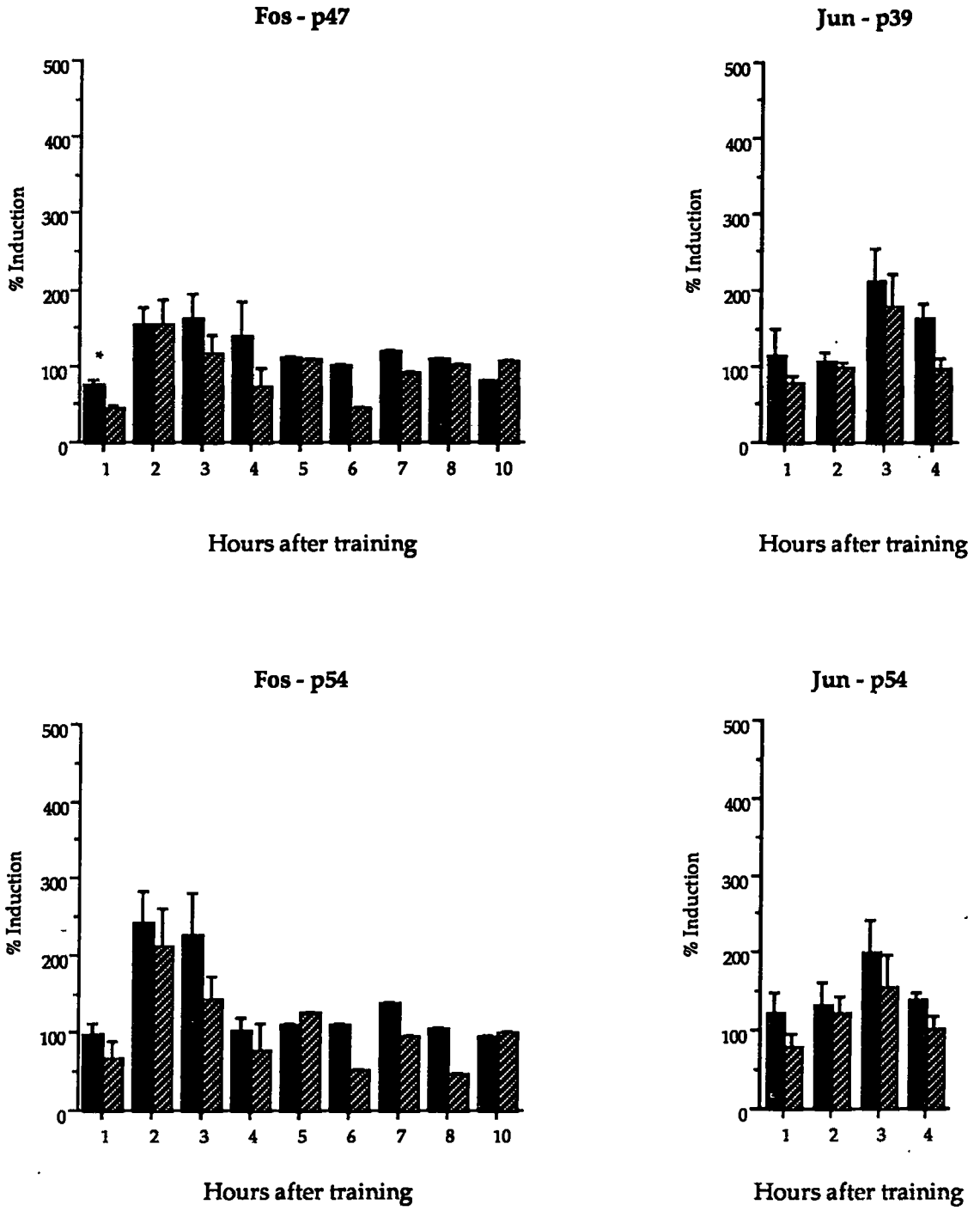
There was an increase in expression of Fos and Jun containing proteins in both the left and right IMHV and LPO of Water and MeA trained birds compared to Quiet controls. Comparisons between MeA-trained chicks and Water-trained birds showed that there was a significant increase in Fos (p47 and p54) and Jun (p39 and p54) in the left IMHV and for Fos (p47) in the right IMHV 1 hour after training on a MeA coated bead (figures 4.3 and 4.4). A more dramatic increase due to MeA training alone was observed in the right LPO 2 hours after training for both species of Fos and Jun proteins (figure 4.6). At 4 hours after training, only the level of Fos (p47) was statistically greater for MeA trained birds compared to Water trained birds in the left LPO (figure 4.5). After this time point, IEGPs returned to low levels.

Figure 4.3



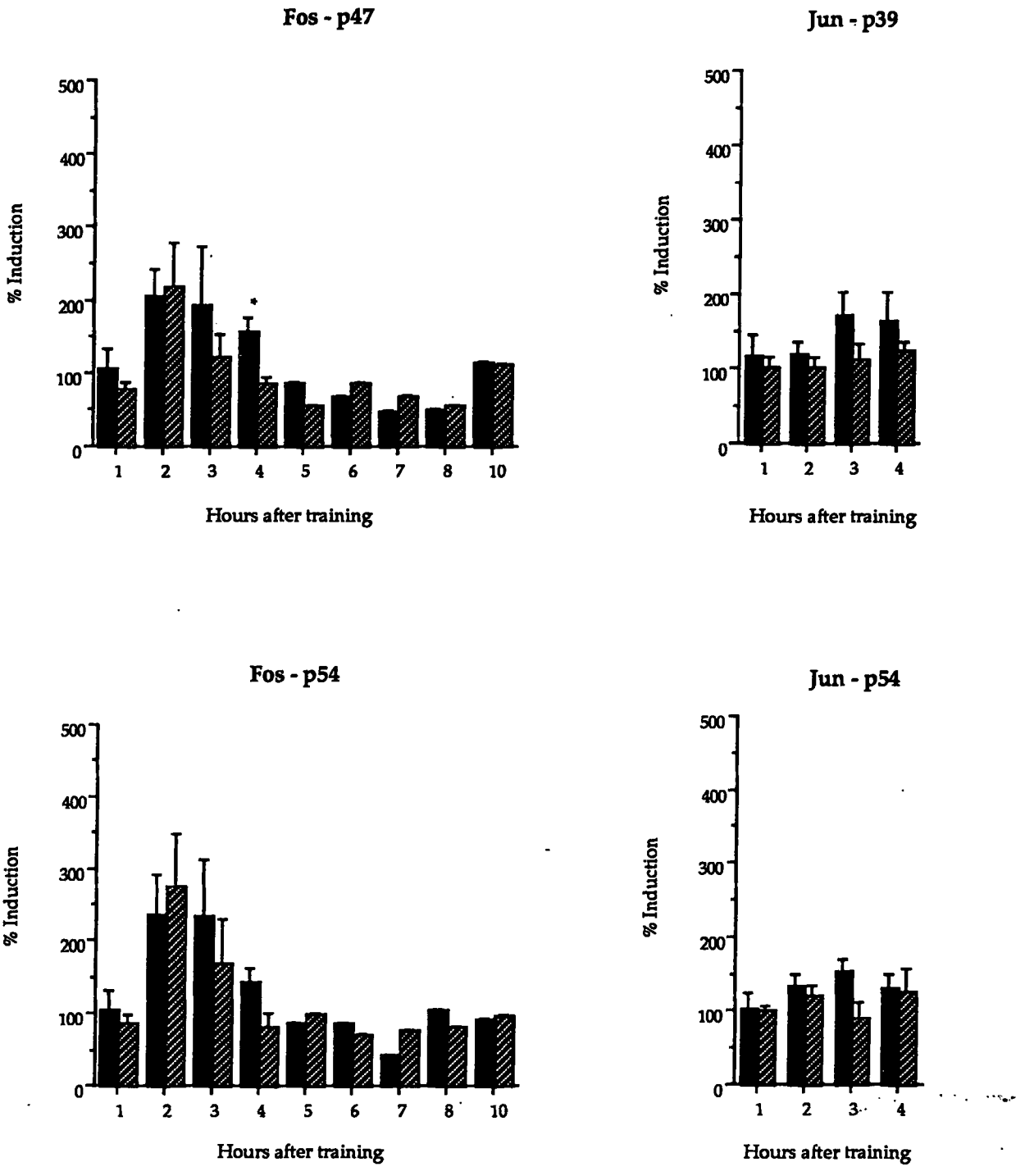
Time course of Fos and Jun proteins induced in the left IMHV after training. Chicks were trained on a MeA or water coated bead. At the times shown chicks were tested for recall. MeA birds that avoided (black bars), Water birds that pecked (hatched bars) and Quiet controls were then killed, and their left IMHV collected for quantification of Fos and Jun levels by Western blotting. Results are expressed as the percentage induction compared to basal levels of expression (Quiet birds) for each densitometric peak. *$p < 0.05$

Figure 4.4



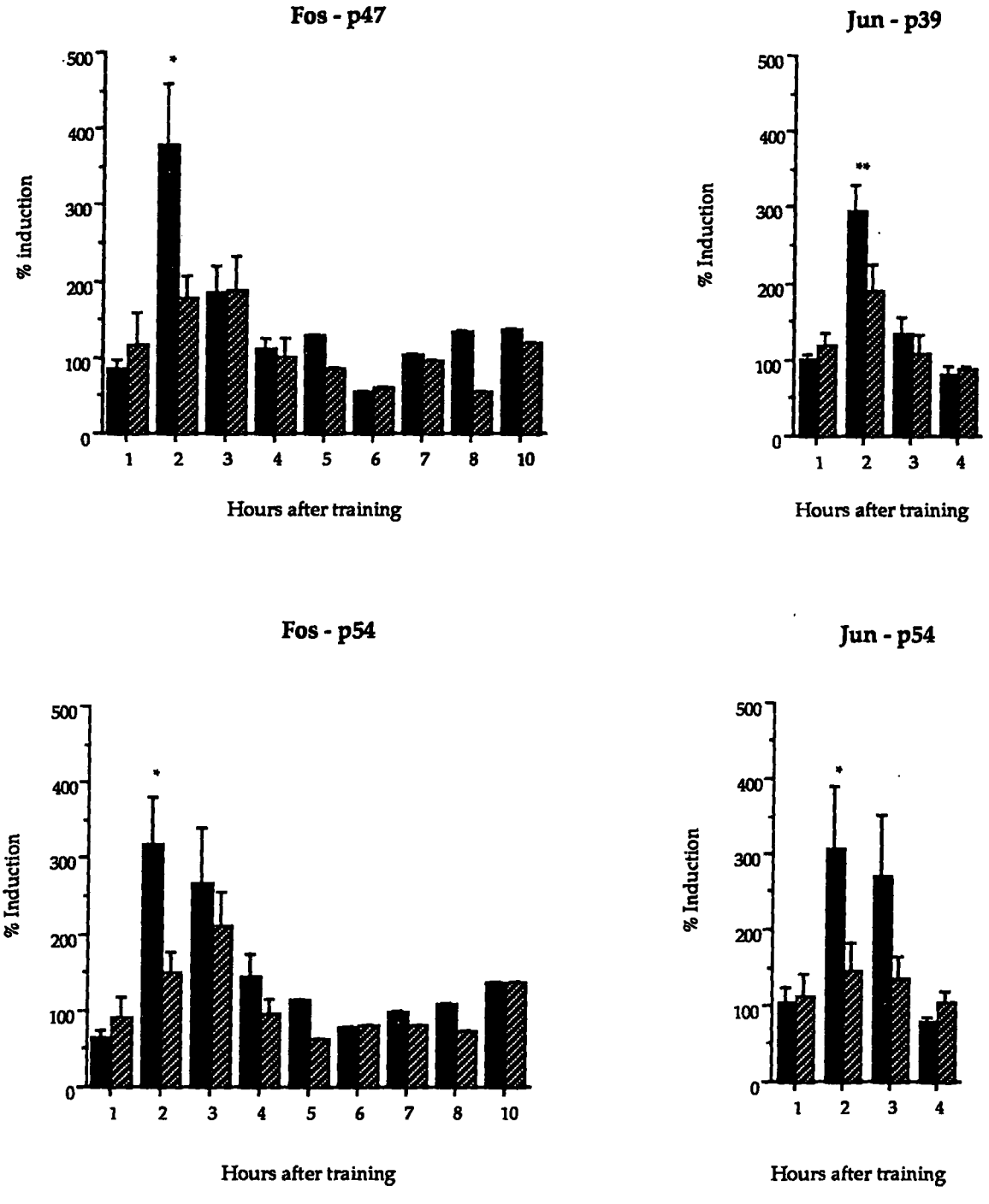
Time course of Fos and Jun proteins induced in the right IMHV after training. Chicks were trained on a MeA or water coated bead. At the times shown chicks were tested for recall, MeA birds that avoided (black bars), Water birds that pecked (hatched bars) and Quiet controls were then killed, and their right IMHV collected for quantification of Fos and Jun levels by Western blotting. Results are expressed as the percentage induction compared to basal levels of expression (Quiet birds) for each densitometric peak. *$p < 0.05$

Figure 4.5



Time course of Fos and Jun proteins induced in the left LPO after training. Chicks were trained on a MeA or water coated bead. At the times shown chicks were tested for recall. MeA birds that avoided (black bars), Water birds that pecked (hatched bars) and Quiet controls were then killed, and their left LPO collected for quantification of Fos and Jun levels by Western blotting. Results are expressed as the percentage induction compared to basal levels of expression (Quiet birds) for each densiotmetric peak. *$p < 0.05$

Figure 4.6



Time course of Fos and Jun proteins induced in the right LPO after training. Chicks were trained on a MeA or water coated bead. At the times shown chicks were tested for recall. MeA birds that avoided (black bars), Water birds that pecked (hatched bars)and Quiet controls were then killed, and their right LPO collected for quantification of Fos and Jun levels by Western blotting. Results are expressed as the percentage induction compared to basal levels of expression (Quiet birds) for each densitometric peak. $^{**}p < 0.025$; $^{*}p < 0.05$

Experiment 4.3: Identification of regions of chick forebrain associated with Fos/Jun induction following training.

Results from the previous experiment (4.2.) implicated the left IMHV and right LPO with IEG induction during the acquisition and retention of a memory. Since most Fos and Jun proteins induction occurred in the right LPO 2 hours after training, this time point was chosen for study. The aim of this experiment was two-fold; firstly, to use immunocytochemistry to map more precisely the regions in which IEGP induction occurs following training, and secondly, to ascertain if the increase in IEGP was due to "recruitment" of a select number of neurones or whether a small population of neurones were increasing their expression of Fos and Jun proteins.

METHOD

Training protocol, tissue collection and analysis.

As previously described chicks were left to equilibrate over night. The following morning they were trained on a Water or MeA coated bead and tested for recall 2 hours later. Only birds that remembered the training experience, MeA birds that avoided (70%), Water birds that pecked (80%) and Quiet controls were then anaesthetised with Sagatol (phenobarbitone) ready for perfusion, and assayed for Fos and Jun proteins by immunocytochemistry (for method see chapter 3).

Three sections, 20μM thick, and at 200μM intervals, were cut in the region of the LPO and mounted on one slide. 2 replicate slides were also collected, consisting of consecutive sections to the slices already obtained. This protocol was repeated so that sections containing the IMHV were also collected. One of each pair of duplicate slides was then probed for Fos or Jun immunoreactivity; the third slide was retained for specific histological identification of regions.

The number of positively stained nuclei within the field of view of the microscope at a magnification of x40 (101800 μm^2) were counted (nuclear density). Three slices per chick from different depths of each region were probed for Fos or Jun immunoreactivity and a mean nuclear density value was calculated. In addition to nuclear counting in IMHV and LPO, the mean nuclear density was calculated for their HA and HP in each brain. These areas were included as control regions. See fig 3.1 for identification of brain regions.

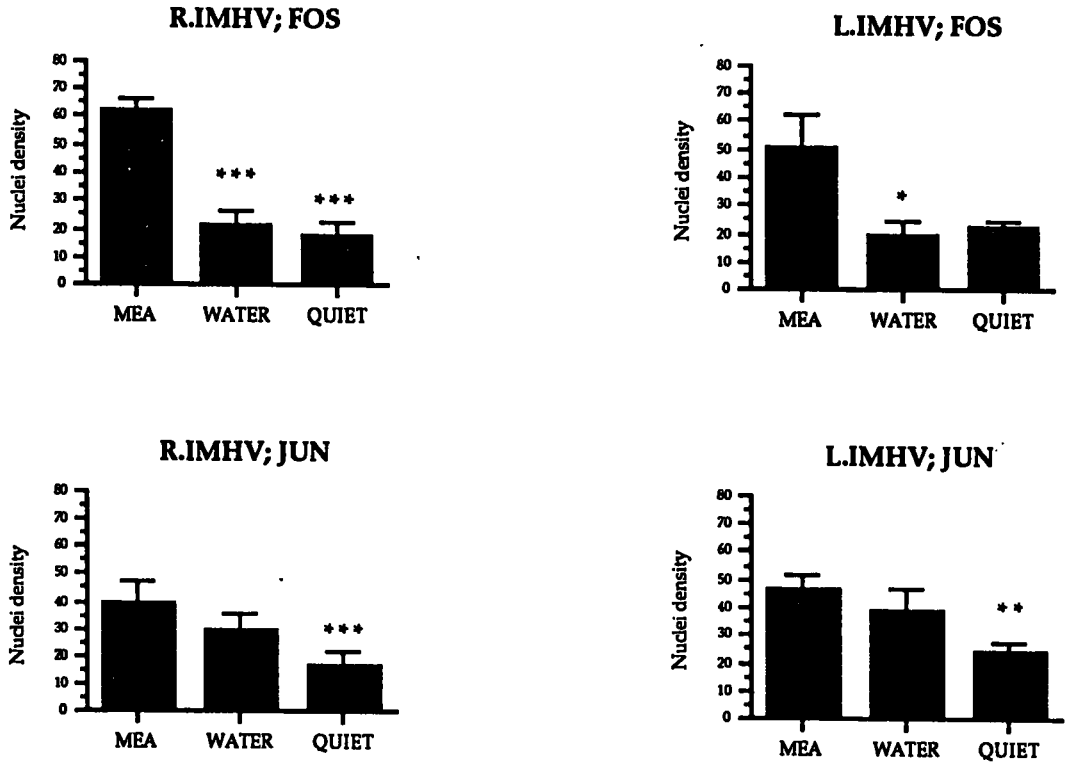
Statistical treatment of data

Comparisons of the density of nuclear staining for each region were made between birds trained on MeA and Water or Quiet chicks using the Mann-Whitney U test.

RESULTS

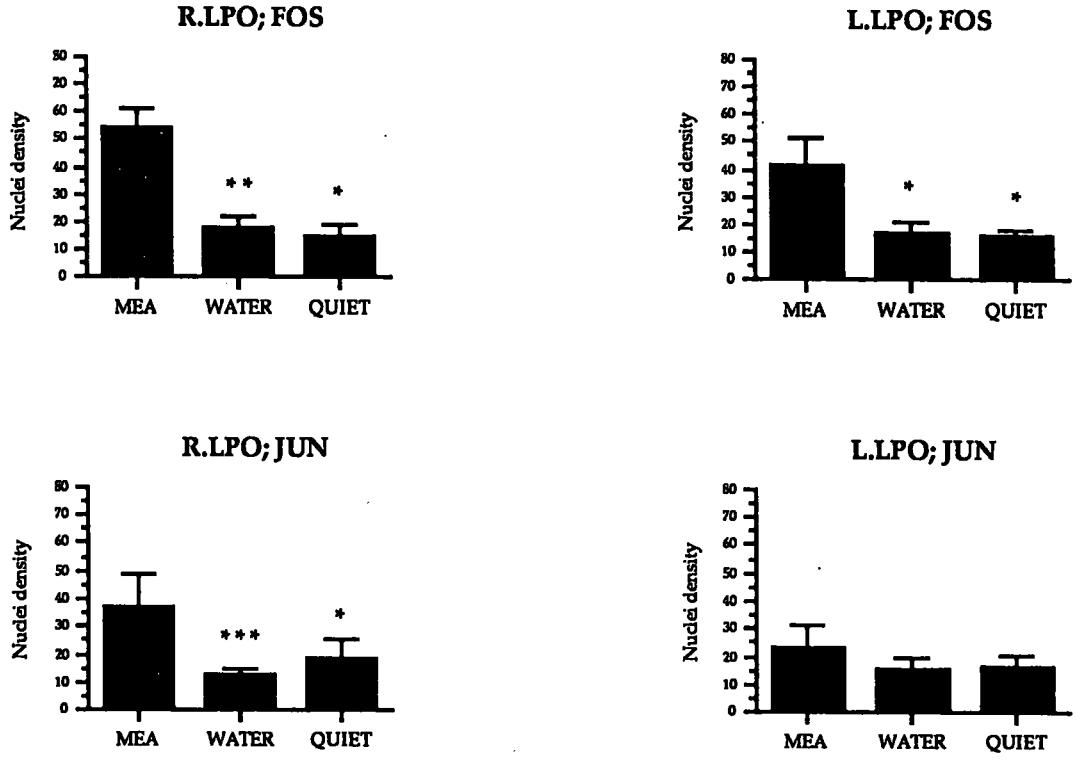
Two hours after MeA training, there was an increase in the number of Fos-positive stained nuclei in both right and left IMHV and LPO compared with Water birds. The density of Fos-positive staining in the Water trained birds was not significantly different from that in untrained Quiet control birds (figs 4.7 and 4.8). Immuno-positive staining for Jun protein was only significantly greater in MeA chicks than Water controls in the right LPO. In both IMHVs, the MeA training experience induced Jun above Quiet levels but not above Water trained chicks. There was no increase in the density of Jun-positive nuclei in the left LPO after either training experience. The other areas studied, HA (figure 4.9) and HP (figure 4.10), did not exhibit any increase in the number of positive stained nuclei when trained on either the MeA or Water bead compared to Quiet controls.

Figure 4.7



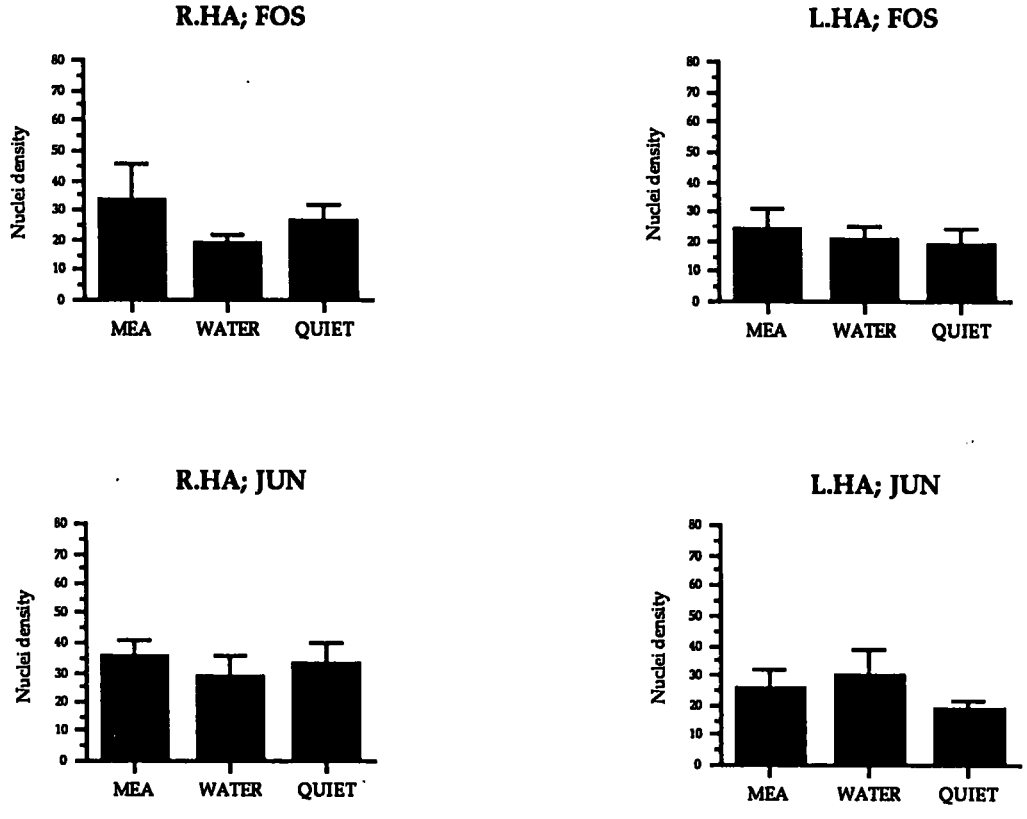
Density of Fos- and Jun- positive nuclei in the IMHV. Chicks were trained on a bead coated with Water or MeA. At 2 hours post-training chicks were tested for recall. MeA birds that avoided, Water birds that pecked and Quiet control birds were assayed for Fos or Jun immunoreactivity. Results are expressed as the mean density of Fos or Jun positive nuclei. ***$p < 0.01$; **$p < 0.025$; *$p < 0.05$

Figure 4.8



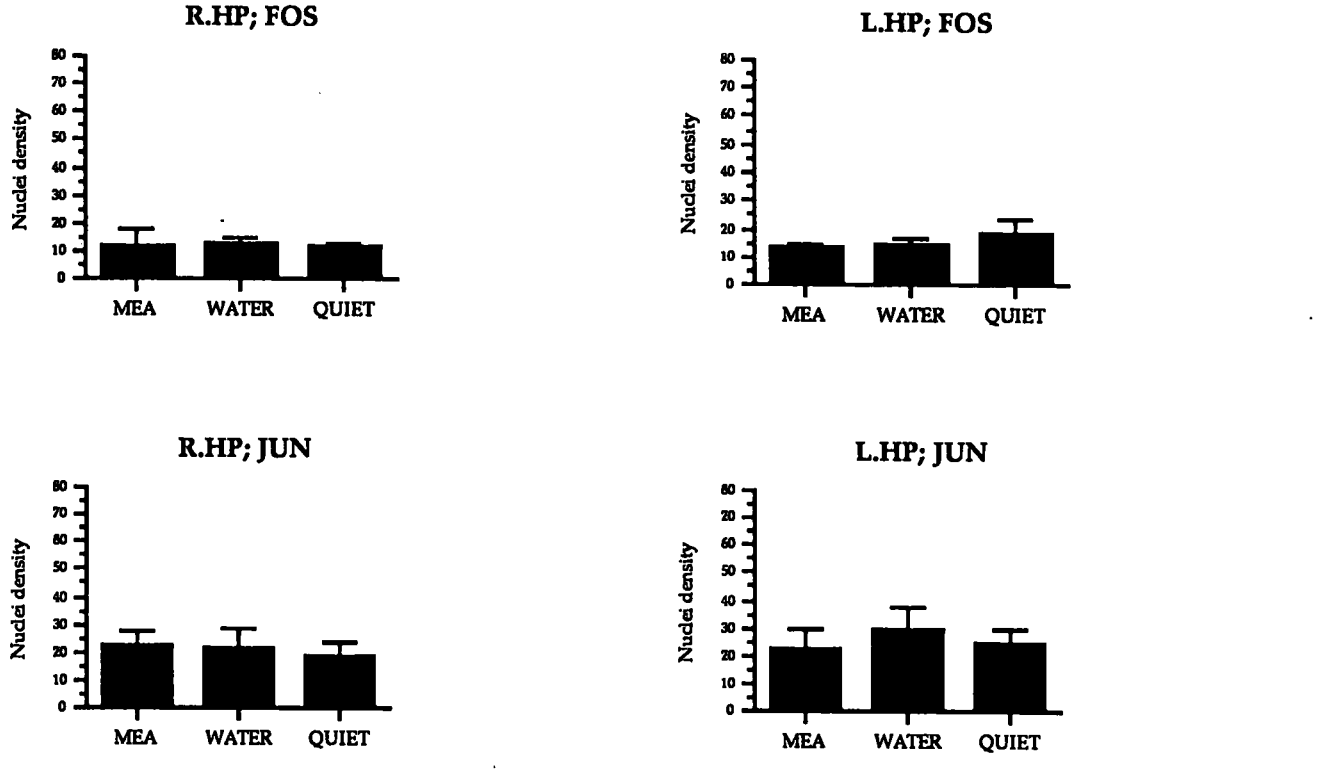
Density of Fos- and Jun- positive nuclei in the LPO. Chicks were trained on a bead coated with Water or MeA. At 2 hours post-training chicks were tested for recall. MeA birds that avoided, Water birds that pecked and Quiet control birds were assayed for Fos or Jun immunoreactivity. Results are expressed as the mean density of Fos or Jun positive nuclei. ***$p < 0.01$; **$p < 0.025$; *$p < 0.05$

Figure 4.9



Density of Fos- and Jun- positive nuclei in the HA. Chicks were trained on a bead coated with Water or MeA. At 2 hours post-training chicks were tested for recall. MeA birds that avoided, Water birds that pecked and Quiet control birds were assayed for Fos or Jun immunoreactivity. Results are expressed as the mean density of Fos or Jun positive nuclei.

Figure 4.10



Density of Fos- and Jun- positive nuclei in the HP. Chicks were trained on a bead coated with Water or MeA. At 2 hours post-training chicks were tested for recall. MeA birds that avoided, Water birds that pecked and Quiet control birds were assayed for Fos or Jun immunoreactivity. Results are expressed as the mean density of fos or jun positive nuclei.

DISCUSSION

In spite of the problems of fluctuating Fos levels in Quiet controls, Western blotting showed that all the species of Fos and Jun proteins identified in chapter 3 are induced in response to training in both IMHV and LPO. In all the regions studied, there was little or no increase in the induction of any of the proteins measured compared to Quiet controls one hour after training. This may be because the levels of IEGP in Quiet control were unusually large, masking any increase in the IEGP expression. However, the salient point to be derived from these observations is that, for the left IMHV, there is a statistical difference between the amount of Fos and Jun proteins in MeA- over Water-trained birds. Fos and Jun proteins levels for all regions studied only begin to rise above Quiet controls after 2 hours, peaking in all areas at 3 hours, with the exception of the right LPO, which peaked and showed the greatest level of induction after only 2 hours. Again, this is significantly greater for MeA birds over Water birds. There was no further induction of these proteins after 4 hours, as observed in samples collected between 5 and 10 hours after training. Fos protein is known to be induced maximally at between 30 and 60 minutes after stimulation, and decays with a half-life of 2 hours (Curran and Morgan, 1986; Muller et al., 1984), whereas, Jun peaks at 1 hour and breaks down with a half-life of 1 hour (An et al., 1993). Careful study of the kinetics of induction and decay of the identified species of Fos and Jun show that they concur with Curran and Morgan's (1984) findings. This reinforces the assumption that the bands revealed by immunoblotting are in fact genuine Fos and Jun proteins.

This increase in the right LPO, for both Jun and Fos, was also observed 2 hours post-training by immunocytochemistry. In the left IMHV, results from immunocytochemistry demonstrated that Fos and Jun were induced in response to the MeA beads but this increase was only significant for Fos when compared to Water controls. The number of Jun-positive nuclei in the left

IMHV in response to the Water bead was intermediate between MeA trained and Quiet control levels. This discrepancy in the left IMHV, between data derived from immunocytochemistry and Western blotting could be due to the small sample size for immunocytochemistry. In addition, the significant induction of IEGs was observed by Western blotting at only the 1 hour time point.

In the right IMHV, there was a significant increase in the number of positively-stained Fos nuclei after MeA-training compared with Water-trained chicks. However, for Jun, there was a "step-up" gradation from Quiet to Water to MeA trained. In the left LPO, there was a similar induction of Fos as observed in the right IMHV. This Fos induction in the left LPO was not accompanied by any increase in Jun expression. The results for both the right IMHV and left LPO contradicts those of the Western blotting data. The Western blotting data indicates an approximately equal induction of IEGPs in response to both beads. However, immunocytochemistry revealed induction of Fos in response to the MeA bead only. Again, this could be due to the small sample size employed in the immunocytochemistry method. Alternatively, the nuclear concentration of Fos or Jun transcription factors, and/or the number of neurones required to form a stable memory for each region studied, could be different.

The HA and HP showed no increase in the density of positively stained Fos or Jun nuclei in response to training. This is in agreement with the results of Szekely et al (1992). In the Szekely study, the induction of Fos was measured by immunoctytochemistry (using the same anti-Fos antibody donated by Prof. P.J. Sharp) in marsh tits after food-storing. They showed no increase in the total number of Fos-stained neurones in the HA or HP even though there was an induction in response to the task for both left and right HV and LPO. This initially indicates that the HP is not involved in learning and memory. This is

not true because pretraining lesioning of the HP renders chicks amnesic for the passive avoidance task (Sandi et al., 1992). However, an explanation could be that in the avian brain this region is active but does not require the activation of Fos or Jun. It may be however, that another IEG (eg *krox*24, c-*myc*) is involved. Since measurements for these two regions and the IMHV were made on the same tissue slice, they act as negative controls for the immunocytochemistry method, re-inforcing the fact that IMHV and LPO are active following training.

The Western blotting data shows that Jun levels do not peak until the 3 hour time point for both right IMHV and left LPO, whereas Fos peaks 2 hours post-training. This may account for the increase in the number of Fos-positive nuclei following MeA-training compared with Water-training, which was not observed for Jun. If the degree of nuclei staining was quantified in birds 3 hours after training, then there may be a significant increase in the number of active neurones expressing Jun.

Western blotting and immunocytochemistry showed that the act of pecking a bead induces both Fos and Jun, and increases the number of active nuclei in LPO and IMHV. This implies that other cues from the passive avoidance training paradigm eg. novel stimulation and/or colour, are also encoded within these areas. It is also possible that chicks trained on a Water bead are learning and therefore forming a 'weaker memory trace', by recruiting fewer neurones. They therefore express less IEGPs compared to MeA trained birds who form a 'strong memory trace'. In fact Richardson et al (1992) have demonstrated a correlation between the level of LTP induction and the level of expression of the IEG, *Zif*/268. The amount of Fos and Jun expressed and number of neurones active as a consequence of training on either the MeA or Water bead should be exactly the same for certain aspects of the memory (eg. bead position, colour etc.). Any induction due to the adversive nature of the

stimulus (memory) of the MeA would be over and above this. Hence, it may be possible to dissociate some aspects of the training paradigm in a temporal and spatial manner. A high proportion of the chicks trained on the water bead demonstrated that they had recalled upon testing by running up to the bead and pecking avidly at it. This could represent an appetitive memory, which could be encoded in different regions of the brain to the MeA aversive memory. Thus, one can dissociate, only to a certain extent, the role of one region from another. It is also possible that novel stimulation and "weak learning" are the same, within this paradigm. In spite of these difficulties, MeA training, which is the stronger adversive stimulus compared to Water training, demonstrates that the left IMHV and right LPO were active in adversive memory acquisition and formation.

Radford et al (1981) have shown that there are periods of the day when chicks are more receptive to certain aspects of learning and memory. They used a similar passive avoidance training paradigm, where chicks were subjected to 14:10 hour light/dark cycle for 4 days pre-hatching. This light/dark cycle was maintained throughout training and testing. When the chicks were 1-2 days old they were pretrained on a water coated bead and trained on either a red or blue bead, dipped in either MeA or water, at various times during the day. Chicks were tested 24 hours after training on their ability to discriminate between Water and MeA test beads. The results showed that discrimination was best (for chicks trained and tested) at 1200 hours, with least evidence of generalisation and poorest retention in chicks trained and tested at 1600 hours. This paradigm differed from the one employed here. Chicks received 12:12 hour light/dark 2 days prehatch and were housed under red light for 16 hours before and during training. In addition, the chicks go through periods of inactivity and activity during the training day which is in direct conflict with Radford's findings where chicks were very active during the light period and inactive during the dark period. However, the salient point is that they did

find periods of the day when chicks were more responsive to certain aspects of learning. These circadian cycles probably involve fluctuations in hormone levels etc. which are known to influence IEG expression. This could account for fluctuations in levels of Fos observed throughout the experimental day (expt. 4.1).

Immediately following training ,Rose and Csillag (1985) showed an increase in the 2-DG uptake in the left IMHV. 30 minutes later, there is an enhanced uptake of Ca^{2+} (believed to be presynaptic) (Clements and Rose, 1994), an upregulation of NMDA receptor activity and a change in the phosphorylation state of the presynaptic PKC substrate B50 in the left IMHV. Pre-training lesions of the left IMHV have implicated this area in the acquisition of long-term memory (Patterson et al, 1990). Also, behavioural pharmacological studies using unilateral intracranial injections of glutamate in its role as an inhibitor of short-term memory formation (Patterson et al, 1986), 7-ClK which antagonises NMDA receptor activation (Steele and Stewart, 1993) or inhibitors of PKC, have implicated the left IMHV with formation of a memory for passive avoidance learning. Electrophysiological studies have shown an increase in neuronal bursting in the left IMHV 3 hours post-training (Gigg et al, 1993). If, as the immunocytochemistry data shows, neurones are recruited in the left IMHV, this increase in number could be reflected in an increase in electrical activity. The finding that Fos and Jun proteins are induced in the left IMHV 1 hour after training adds further credence to the theory that the left IMHV plays a major role in long-term memory formation.

Gilbert et al (1991) suggested that once information regarding a memory had been acquired by the left IMHV, it was rapidly redistributed to the right IMHV and LPO. Electrophysiological studies have shown that there is an increase in bursting rate in right LPO between 4 and 7 hours after testing (Gigg et al., 1993). Again, this could be due to an increase in neuronal recruitment in this area.

Lesion studies also implicate the LPO in the retention of a memory (Gilbert et al, 1991). The results reported here where Fos (p47 and p54) and Jun (p39 and p54) proteins show the greatest induction and neuronal activity in response to learning 2 hours after training in the right LPO, concur with this retention role. The Water- and MeA- training experience induce both species of Fos and Jun proteins studied to the same degree, above Quiet controls in the right IMHV and left LPO. This strongly implicates both structures in aspects of long-term memory formation.

CHAPTER 5

The NMDA receptor and learning-induced IEG expression

INTRODUCTION

Excitatory amino acid (EAA) receptors can be divided into two groups: ionotropic and metabotropic. Ionotropic receptors are coupled to ion channels and increase the ionic concentration within the cell by allowing an influx of extracellular ions such as Ca^{2+} into the post-synaptic cell. Metabotropic receptors are linked to phosphoinositide breakdown and mobilize intracellular Ca^{2+} from internal stores. The main endogenous agonists of the EAA receptors are glutamate and aspartate. The synthetic agonist, N-methyl-D-aspartate (NMDA), has lead to a simple first-order classification of EAA receptors as either NMDA or non-NMDA receptors (for recent review see Farooqui and Horrocks, 1991).

It has been shown that NMDA receptor activation is critical in various types of synaptic plasticity, such as enhanced synaptic transmission in the visual cortex (Artola and Singer, 1987; Kleinschmidt et al., 1987), the kindling model of epilepsy (Peterson et al., 1983; Mody and Heinemann, 1987), and vestibular compensation after unilateral labyrinthectomy (Smith and Darlington, 1988). One of the most extensively studied models of synaptic plasticity involves the activation of NMDA receptors and the induction of long-term potentiation (LTP) in the CA1 region of the rat hippocampus (Collingridge et al., 1983; Harris et al., 1984). This simple model of LTP induction is believed to be triggered by the "switching-on" of the NMDA-receptor which results in an influx of calcium into the post-synaptic cell (Bliss and Collingridge, 1993; Madison et al., 1991). However, this model of plasticity appears to be more complicated than first thought. Recent investigations into hippocampampal

LTP have indicated that activation of the NMDA receptor did not necessarily lead to the induction of LTP, but instead may have elicited a repertoire of distinct forms of synaptic plasticity. These included short-term potentiation (STP) or long-term depression (LTD). Furthermore, additional mechanisms may exist that inhibit LTP even after low levels of NMDA-receptor activation (for recent review see Malenka and Nicoll, 1993).

The NMDA receptor has also been implicated in learning and memory formation. Eight and a half hours after imprinting chicks on a flashing light, the density of NMDA receptors is increased in the IMHV (McCabe and Horn 1988). Also, studies utilising the passive avoidance learning paradigm show that in the chick, within 30 minutes of training, there is an increase in NMDA receptor binding (Stewart et al., 1992). The NMDA antagonist, MK-801, is considered to bind to a site in the open NMDA channel, so physically preventing the influx of Ca^{2+} through the receptor channel. If MK-801 is injected around the time of training, the chick is rendered amnesic for the task (Burchuladze and Rose, 1990). Similarly, blockade of the glycine site of the NMDA receptor by 7-chlorokynurenate (7-ClK) injected into the left IMHV, results in amnesia (Steele and Stewart, 1993). By contrast, no amnesia results if non-NMDA glutamate receptors are blocked by antagonists such as CNQX (Burchaladze and Rose, 1992).

Fos expression has been shown to be mediated by the activation of NMDA receptor in response to a variety of stimuli, e.g. light (Abe et al., 1991), noxious agents (Aronin et al., 1991), and in a model of brain injury (Herrera and Robertson, 1990; Olenik et al., 1991). If NMDA activation leads to enhanced Fos and Jun expression in the sequence of molecular events following training and is necessary for memory formation, then blockade of the NMDA receptor should not only cause amnesia but also prevent the training-induced increase in IEGP expression. Such studies are complicated by the observation that

intracerebral injections themselves can result in elevated Fos expression (own observation). This problem can be circumvented using the antagonist MK-801, which can cross the blood brain barrier and allows the use of intraperitoneal injections which have been shown previously to produce amnesia (Burchuladze and Rose, 1992). However, the possibility that MK-801 may itself induce Fos (Dragunow and Faull, 1990; Hughes et al., 1993), needs to be controlled for.

The experiments reported here are designed to test the hypothesis that MK-801 blockade of NMDA receptor activity results in amnesia and abolishes learning-related IEGP expression, previously observed in left IMHV 1 hour and right LPO 2 hours post-training (chapter 4).

METHODS

Training and tissue collection

As usual, on the day of hatching, birds were put into pens and left overnight to equilibrate. This ensured that Fos and Jun proteins had fallen to low levels. At 09.00h the following morning, chicks were trained on an MeA bead according to the one-trial passive avoidance paradigm (chapter 3). Five minutes post-training, chicks received intraperitoneal (i.p.) injections of either 0.2 ml saline (0.9% NaCl) or 0.2ml MK-801 (75mM in 0.9% saline). The remaining Quiet control groups were left undisturbed throughout the whole experiment. At 1 or 2 hours post-training, birds were tested and scored for recall of the task as either peck or avoid. At test, regardless of the score, all birds, including controls for that time point, were decapitated. For the 1 hour time point, the left and right IMHV were collected and for the groups tested 2 hours after training the right LPOs were removed. As in previous experiments, it was necessary to pool tissue from two birds for each condition and test. Western blotting was carried out as previously described (chapter 3).

Experiment 5.1. The effect of MK-801 on recall 1 or 2 hours after training.

Burchuladze and Rose (1992) demonstrated that i.p. injections of MK-801 (0.5mg/Kg) rendered chicks amnesic for the training task when tested 3 hours later. They did not show amnesia 30 minutes after training. No intermediate times were tested, but the authors argued that NMDA receptor was essential for long-term memory formation. The first experiment reported in this chapter tests whether i.p. injections of MK-801 cause amnesia for the training task 1 and 2 hours post-training.

METHOD

Chicks were trained on the MeA bead, injected with MK-801 (0.5mg/Kg) or Saline and tested for recall of the experience 1 or 2 hours post-training (n: 1 hour, Saline = 16, MK-801 = 18; 2 hours, Saline = 16, MK-801 = 16).

Statistical treatment of data

Behavioural scores were expressed as the percentage of birds that exhibited recall (% avoidance) for the training experience on test 1 or 2 hours after training. Comparisons between MK-801 and Saline injected birds were made using χ^2.

RESULTS

Intraperitoneal injections of MK-801, 5 minutes post-training, rendered chicks amnesic for the task 1 or 2 hours later. The results of the experiment are represented graphically in figure 5.1.

Figure 5.1

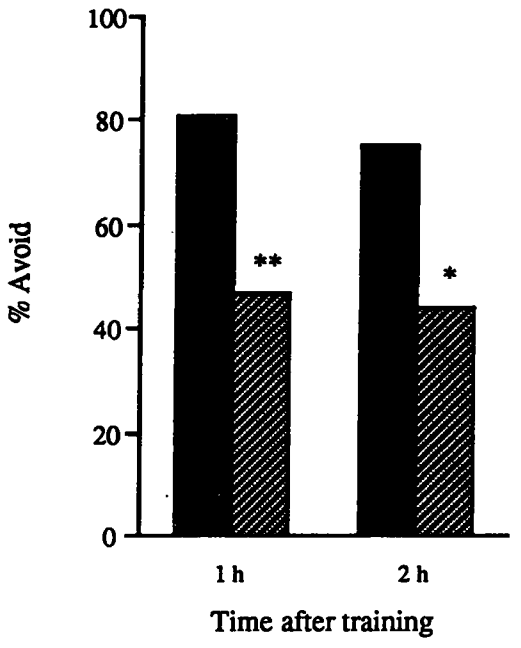


Figure 5.1; The effect of MK-801 on recall 1 and 2 hours after training. Chicks received intraperitoneal injections of 0.15 mMoles of MK-801 in 0.2 mls of saline (hatched histograms), or saline (filled histograms), 5 minutes after passive avoidance training. Recall was tested for 1 or 2 hour later. *, $p<0.02$; **, $p<0.01$.

Experiment 5.2. The effect of MK-801 on the expression of Fos and Jun proteins 1 or 2 hours post training.

The aim of this experiment was to determine whether any correlation exists between amnesia produced by blockade of the NMDA receptor by MK-801 and inhibition of IEGP induction. As the previous chapter (4) showed that MeA training induced Fos and Jun proteins in the left IMHV 1 hour, and the right LPO, 2 hours after training, both left and right IMHV were collected at 1 hour and the right LPO 2 hours, after training and MK-801 injection.

METHOD

The left and right IMHV 1 hour, and the right LPO 2, hours post-training were collected from chicks used in experiment 5.1. Paired samples were then assayed for Fos and Jun proteins by Western blotting. This resulted in four groups of animals (not including Quiet birds) for each time point and region; MK-801-peck (n= 7-8), MK-801-avoid (n= 7-8), saline-peck (n = 3) and saline-avoid (n= 11-12).

Statistical analysis of data

Individual densitometric peaks were expressed as the percentage induction for individual proteins compared to basal levels (Quiet birds) between samples run on the same gel. For comparisons, birds were divided into four groups: MK-801 or Saline injected, peck or avoid. Statistical comparisons were made for each protein species expressed between groups using Mann-Whitney two-tailed, U test.

RESULTS

Both MK-801 and Saline injected birds that showed recall for the task, by avoiding the previously bitter bead on test, showed higher levels of IEGP

expression than those that were amnesic for the task. The amnesic groups of injected birds did not show any significant induction of IEGP above Quiet control birds. Compared with amnesic MK-801 injected birds induction of IEGP in chicks with recall for the training experience was significantly higher in MK-801 injected birds 1 hour after training, in the right IMHV for Jun-p39 and in the left IMHV for Fos-p47, Jun-p39 and p54. Similarly, in saline injected birds that showed recall compared to saline injected amnesic birds, there was a significant induction of Jun-p39 and p54 in the right IMHV, and Jun in the left IMHV. In the right LPO 2 hours after training, significant differences between birds that pecked and those that avoided were seen, with MK-801 injected birds for Fos-p47 and p54, and Jun-p39 and p54, and in Saline injected birds for Fos-p54, Jun-p39 and p54. The results of the experiment are represented graphically in figures 5.2. - 5.4.

Figure 5.2. The effect of MK-801 on expression of Jun and Fos proteins 1 h after training in the right IMHV. Chicks received i.p. injections of MK-801 (0.15 mMoles) or 0.9% saline 5 minutes after training. 1 hour later they were tested and scored for recall, killed and their right IMHV collected (MP = MK-801/peck, MA = MK-801/avoid, SP = saline/peck, SA = saline/avoid). Fos (p47 and p54) and Jun (p39 and p54) concentrations were expressed as percentage increase with respect to Quiet birds. Comparisons within each treatment group were made between birds that pecked and birds that avoided. *$p<0.05$; ***$p<0.01$.

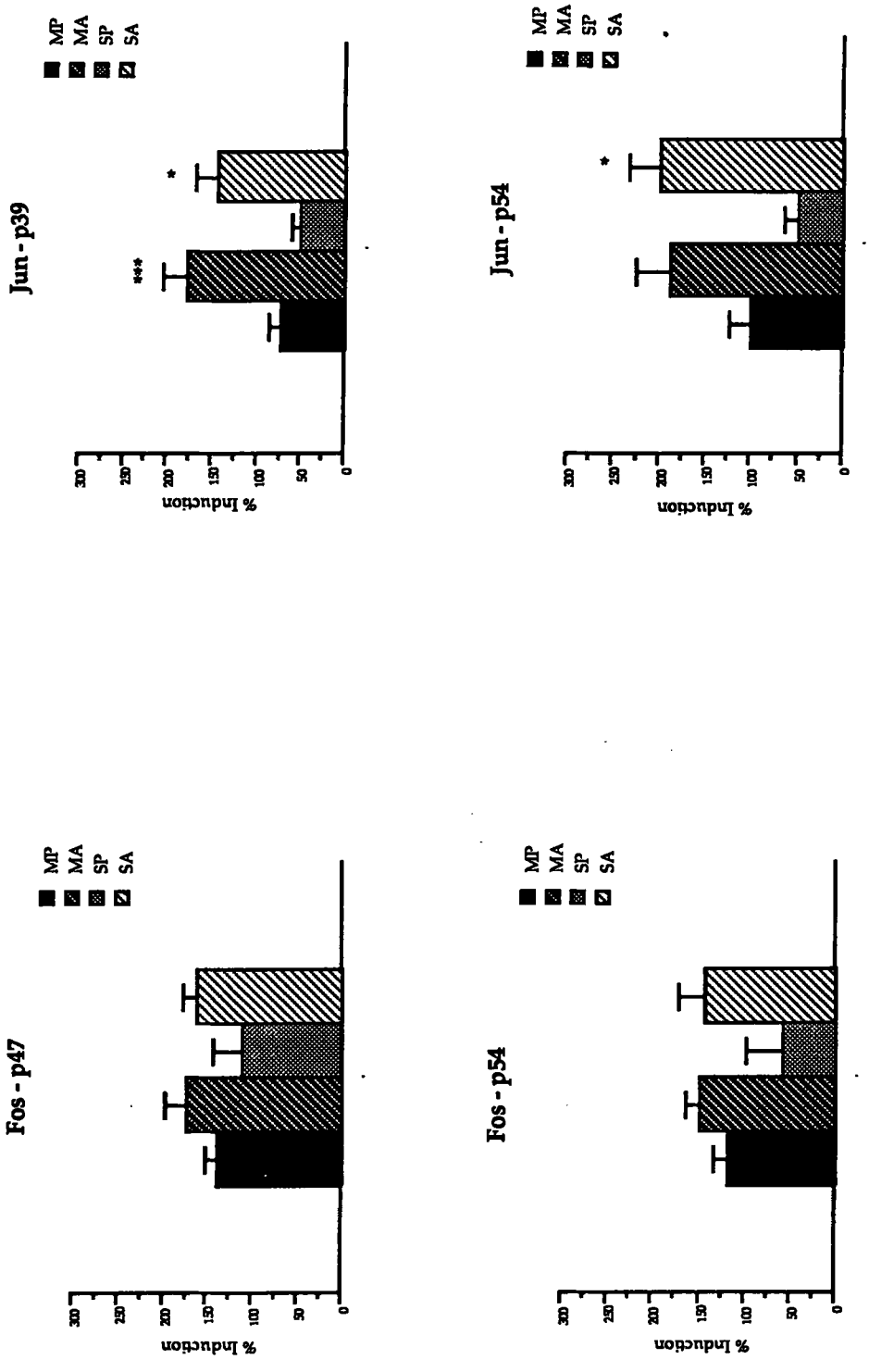

Jun - p39
MP
MA
SP
SA
***
*
% Induction
300
250
200
150
100
50
0
Jun - p54
MP
MA
SP
SA
*
% Induction
300
250
200
150
100
50
0
Fos - p47
MP
MA
SP
SA
% Induction
300
250
200
150
100
50
0
Fos - p54
MP
MA
SP
SA
% Induction
300
250
200
150
100
50
0

Figure 5.2

Figure 5.3. The effect of MK-801 on expression of Jun and Fos proteins 1 h after training in the left IMHV. Chicks received i.p. injections of MK-801 (0.15 mMoles) or 0.9% saline 5 minutes after training. 1 hour later they were tested and scored for recall, killed and their left IMHV collected (MP = MK-801/peck, MA = MK-801/avoid, SP = saline/peck, SA = saline/avoid). Fos (p47 and p54) and Jun (p39 and p54) concentrations were expressed as percentage increase with respect to Quiet birds. Comparisons within each treatment group were made between birds that pecked and birds that avoided. *$p<0.05$; **$p<0.02$; ***$p<0.01$.

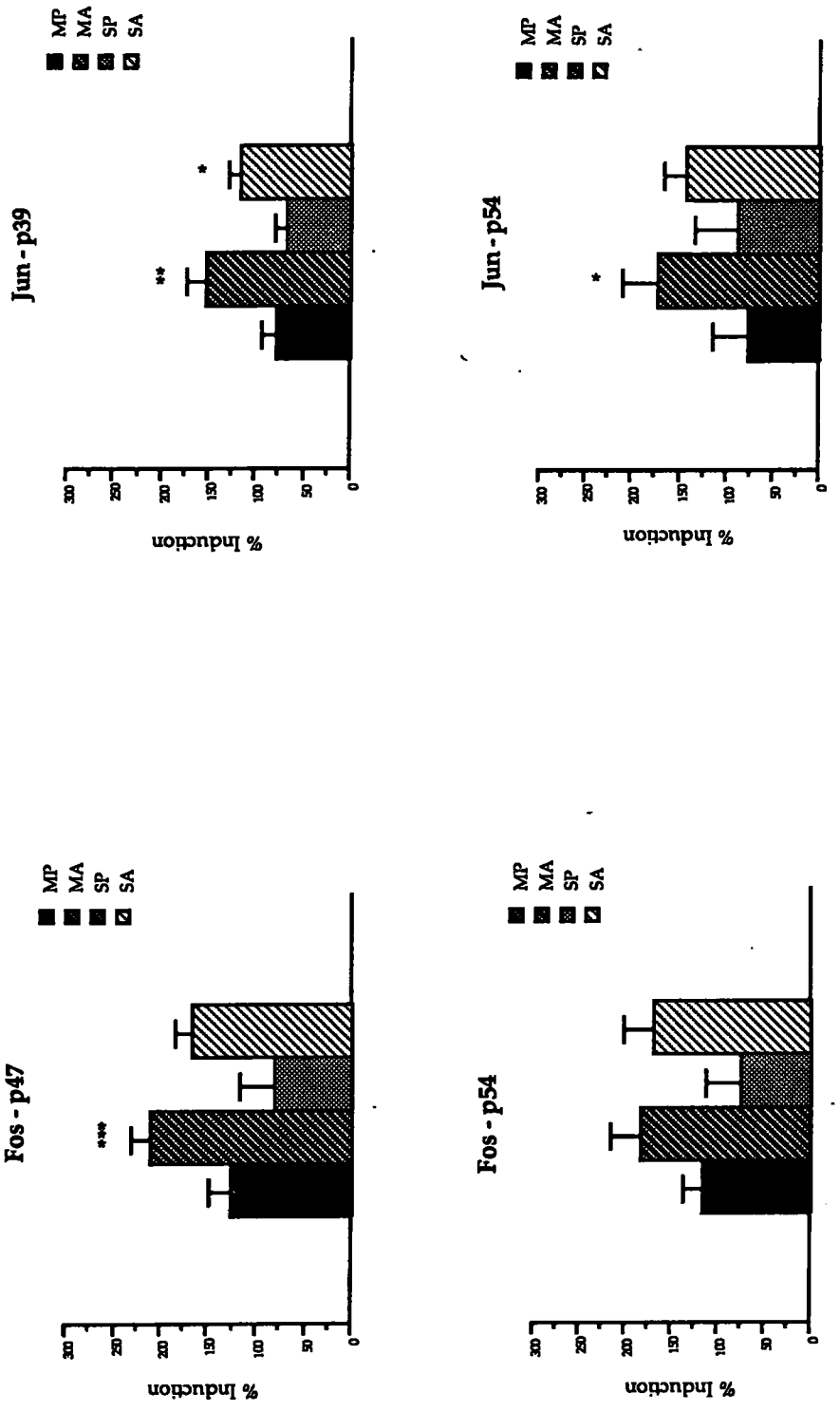
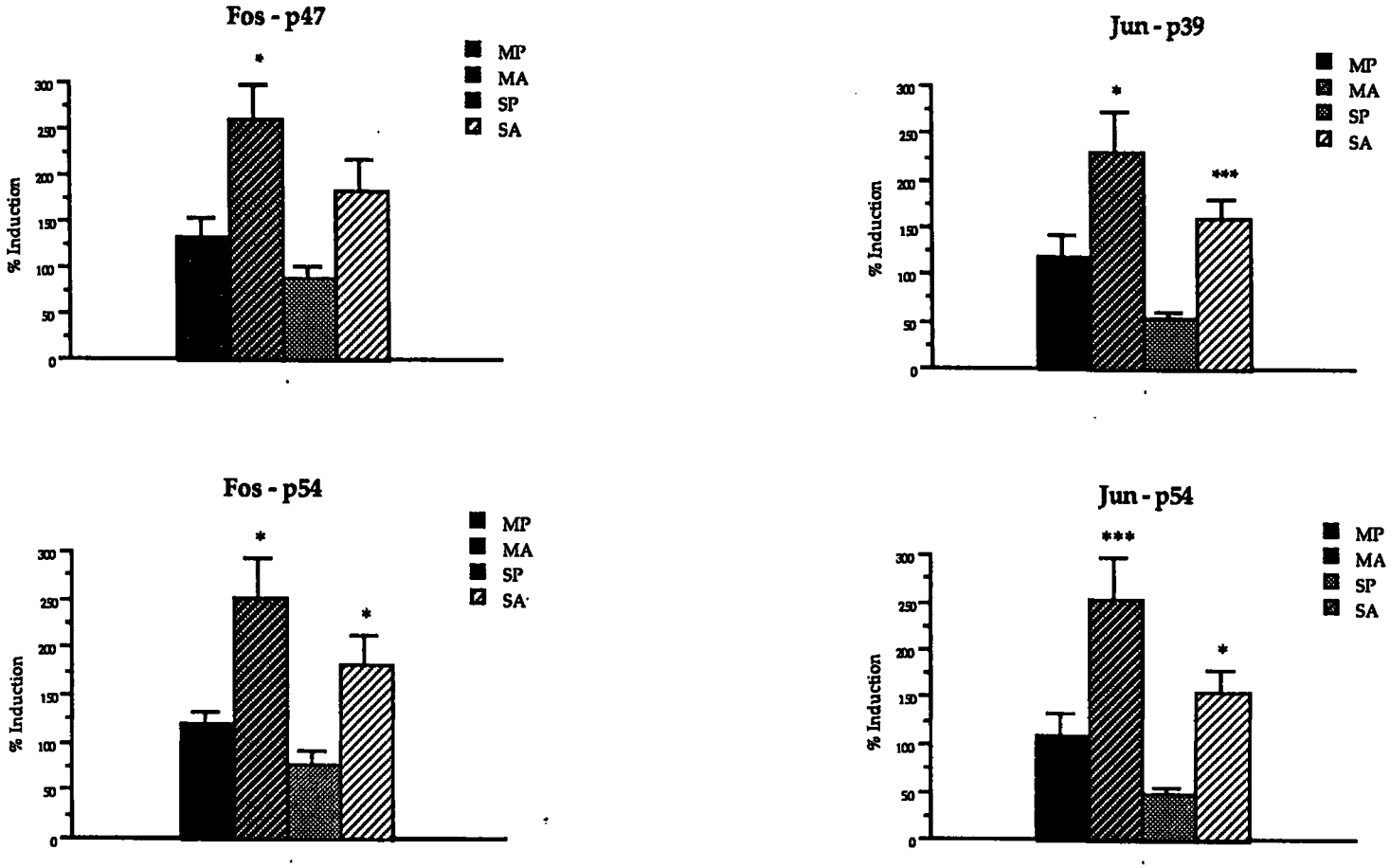

Jun - p39
Jun - p54
Fos - p47
Fos - p54
MP
MA
SP
SA
% Induction
0
50
100
150
200
250
300
*
**
***

Figure 5.3

Figure 5.4. The effect of MK-801 on expression of Jun and Fos proteins 2 h after training in the right LPO. Chicks received i.p. injections of MK-801 (0.15 mMoles) or 0.9% saline 5 minutes after training. 2 hour later they were tested and scored for recall, killed and their right LPO collected (MP = MK-801/peck, MA = MK-801/avoid, SP = saline/peck, SA = saline/avoid). Fos (p47 and p54) and Jun (p39 and p54) concentrations were expressed as percentage increase with respect to Quiet birds. Comparisons within each treatment group were made between birds that pecked and birds that avoided. *$p<0.05$; ***$p<0.01$.

Figure 5.4


Fos - p47
Jun - p39
Fos - p54
Jun - p54
MP
MA
SP
SA
% Induction
0
50
100
150
200
250
300
*
***

DISCUSSION

Generally the data confirms that in chicks which recall the MeA bead, there is also an increase in the amount of all species of Fos and Jun proteins studied in IMHV 1 hour, and right LPO 2 hours, after training (chapter 4). In the group injected with Saline, but which on test were amnesic, there was no increase in induction of these proteins. MK-801 caused amnesia for the task at 1 and 2 hours post-training. In this amnesic group, MK-801 attenuated the induction of Fos, Jun and their respective proteins.

In chapter 4 it was shown that, in the right IMHV, the induction of Fos (p47 and p54) and Jun (p39 and p54) proteins due to the MeA experience was not significantly greater when compared to the Water training experience. MK-801 did not significantly attenuate any induction due to training in birds that recalled the experience. This suggests that induction due to MeA (or Water) training in the right IMHV is not dependent upon the NMDA receptor. Bullock et al (1993) showed that there is a predominance of AMPA receptors in this area compared to the left IMHV, which is rich in NMDA receptors. So, induction here could be mediated via this receptor

Intraperitoneal injections *per se*, do not cause IEGP induction, since the percentage induction of all proteins studied are similar for saline-recall birds to that of uninjected MeA-recall animals (figs. 4. 3-6). However, MK-801-recall birds did have an increase over and above that of Saline-recall birds. These findings are in agreement with those of Dragunow and Faull (1990), who demonstrated that MK-801 induced IEGP in certain regions of the rat forebrain. This induction could be via some non-specific effect of the drug that is related or unrelated to its blockade of the open NMDA receptor.

As stated in the introduction, Ca^{2+} has a ubiquitous role in the cascade of events leading to Fos and Jun expression. Therefore, further work is warranted regarding the exact mechanism(s) by which Ca^{2+} induces them. Since the glycine regulatory site of the NMDA receptor has been implicated in memory formation for the task by its antagonist 7-ClK, perhaps this compound should be employed to corroborate these results.

CHAPTER 6

The inhibition of long-term memory formation by anisomycin

INTRODUCTION

Behavioural pharmacologists have studied the effect of inhibition of cerebral protein synthesis in many types of behaviour and several different animal models (eg. Patterson et al, 1986; Squire et al, 1972a; Flood et al 1972). *De novo* protein synthesis is important in the formation of long-term memory (Barondes and Squire, 1972). Barondes and Squire (1972) further suggested that the proteins involved in neuronal interaction could be:

1) enzymes that regulate the synthesis or destruction of neurotransmitters;

2) proteins that form receptor complexes on the post-synaptic neurone;

3) structural proteins; or

4) proteins which direct specialized types of intracellular recognition (Davis and Squire, 1984).

Antibiotics which act by inhibiting protein synthesis have been used extensively in studies of learning and memory in the chick, mouse and rat (Patterson et al, 1986; Grecksch et al, 1980; Flood et al, 1974; Bradley and Galal, 1987). However, these drugs exhibit powerful non-specific effects. In order to disassociate these non-specific effects, antibiotics that have different sites of action have been employed (Squire and Barondes, 1974). These drugs share the ability to inhibit protein synthesis whilst demonstrating quite distinct non-specific effects. Thus, the amnesic properties of antibiotics such as Anisomycin, Cycloheximide, Puromycin or Acetoxycycloheximide, can be attributed directly to inhibition of protein synthesis (Davis and Squire, 1984).

Puromycin is the least useful for studies of memory formation since it causes hippocampal seziure, swelling of mitochondria and disaggregation of ribosomes (Kerkut et al, 1970, Squire et al, 1972). Cycloheximide also has its drawbacks; it makes animals ill, causing alterations in locomotor activity of mice exposed to an open field (Squire et al, 1970) and it affects the time taken for a mouse to enter the training (electro-shock) chamber in step-through passive avoidance apparatus (Flood et al, 1972).

The antibiotic employed for the following set of experiments was anisomycin (ANI). ANI has been shown to (i) prevent the formation of peptide bonds on the growing amino-acid chain, and (ii) inhibit the enzyme peptidyl transferase (Merck index, 1993). There appears to be some discrepancy in the literature as to the non-specific effects of ANI; earlier studies in the mouse, indicate that the drug has no effect on the animals' locomotion after a discrimination learning task (Flood et al, 1973), whilst others using the passive avoidance training paradigm in the day-old chick have described contrary findings (Patterson et al., 1988; Bradley and Galal 1988).

A major problem associated with behavioural pharmacology is state-dependent learning (Wright, 1974). This phenomenon is well-documented in both animals and humans. If a task is learned under certain drug induced states, the retrieval is best accomplished when the subject is tested while in the same drug state. A memory in this case is impaired if the test is conducted with out drug administration. If a drug has been given pre-training, failure to perform a test could be due either to true amnesia (failure to form memory) or to state-dependency. In the former case, the memory trace is either weakened or absent; in the latter case, the memory is present and can only be reinstated through a return to the same drug state as that obtained at training. This is an important consideration in these experiments since it has been demonstrated in the mouse that inhibition of protein synthesis by ANI lasts for only 2 hours,

after which time protein synthesis resumes (Flood et al, 1973). If this time window of action is similar in the chick, then state dependent learning could be a problem when testing for recall after 24 hours. However, Lee et al. (1989) and Patterson et al (1989) have demonstrated that ANI-induced amnesia in the day-old chick following passive avoidance training is not due to state-dependent learning.

In long-term memory formation, one consequence of protein synthesis is thought to be the synthesis of glycoproteins, such as NCAMs. The primary role that NCAMs play in memory formation is believed to be in synapse modification, resulting in synapse strengthening. 2-deoxygalactose (2-Dgal) inhibits fucosylation of growing sugar chains and hence glycoprotein synthesis. Two periods of amnesia have been observed in the chick following injections of 2-Dgal at different times after training. The first wave of amnesia is apparent when injections of 2-Dgal are administered at between 0 and 2 hours after training; this is thought to be important in memory formation. The second wave of amnesia occurs when 2-Dgal is injected between 5 and 8 hours post-training. Crowe et al (1994) have suggested that during this first wave of neuronal activity in the chick it is existing proteins and not newly synthesised proteins that are fucosylated, since amnesia due to 2-Dgal (45 minutes) occurs before that due to ANI (60 minutes). In addition, it has been shown that inhibition of protein synthesis by cycloheximide immediately after training does not prevent the uptake of radiolabelled fucose (Rose and Harding, 1984). The site of this second wave is regarded as being different from that of the first wave (Scholey et al 1993; 1994). Lesion and electrophysiological studies suggest that the site of the first wave maybe the left IMHV; the site of the second wave could be the LPO or its surrounding areas. As yet these exact locations are undefined (Mason and Rose, 1987; Gigg et al, 1993; Patterson et al, 1990; Gilbert et al, 1991).

The aims of the following experiments were four-fold. Firstly, to investigate the effect of ANI injections at various times relative, to passive avoidance training on the chicks' ability to recall the task. Secondly, to determine in which hemisphere protein synthesis occurs during both the acquisition and retention phases of a long-term memory. Thirdly, to establish the degree of protein synthesis inhibition by ANI, using the expression of Fos and Jun after passive avoidance training and ANI administration as a measure. Fourthly, to determine whether protein synthesis precedes, follows or coincides with glycoprotein synthesis during the second wave of glycoprotein synthesis.

METHODS

Training protocol

For all the behavioural pharmacology experiments day-old chicks were placed in pairs into training pens, with an ample supply of chick crumb. After a one hour period of equilibration, the chicks were ready for passive avoidance training as previously described.

Preparation and injection of drugs

ANI was prepared fresh by dissolving in a minimal quantity of 3M HCl, after which the pH was returned to 7 by the addition of 3M NaOH. The solution was finally adjusted to its working concentration with saline. 2-Dgal was dissolved in saline. Intracranial injections of the drugs in 10 µl per hemisphere were administered directly into the IMHV. The site and depth of delivery were controlled by the use of a specially designed head holder and a sleeved Hamilton syringe (Davis et al, 1979). The chicks were left overnight with food and water. 24 hours post-training, the chicks were tested for recall using a dry chrome bead.

Statistical analysis of behavioural data

Results were expressed as the percentage of birds that exhibited recall (% avoidance) for the training task on test at 24 hours. Comparisons between ANI and saline injected birds were made using χ^2.

Experiment 6.1; Time-course of ANI-induced amnesia.

The aim of this experiment was to determine if two putative waves of protein synthesis, similar to that seen with glycoprotein synthesis, are required for the establishment of a long-term memory.

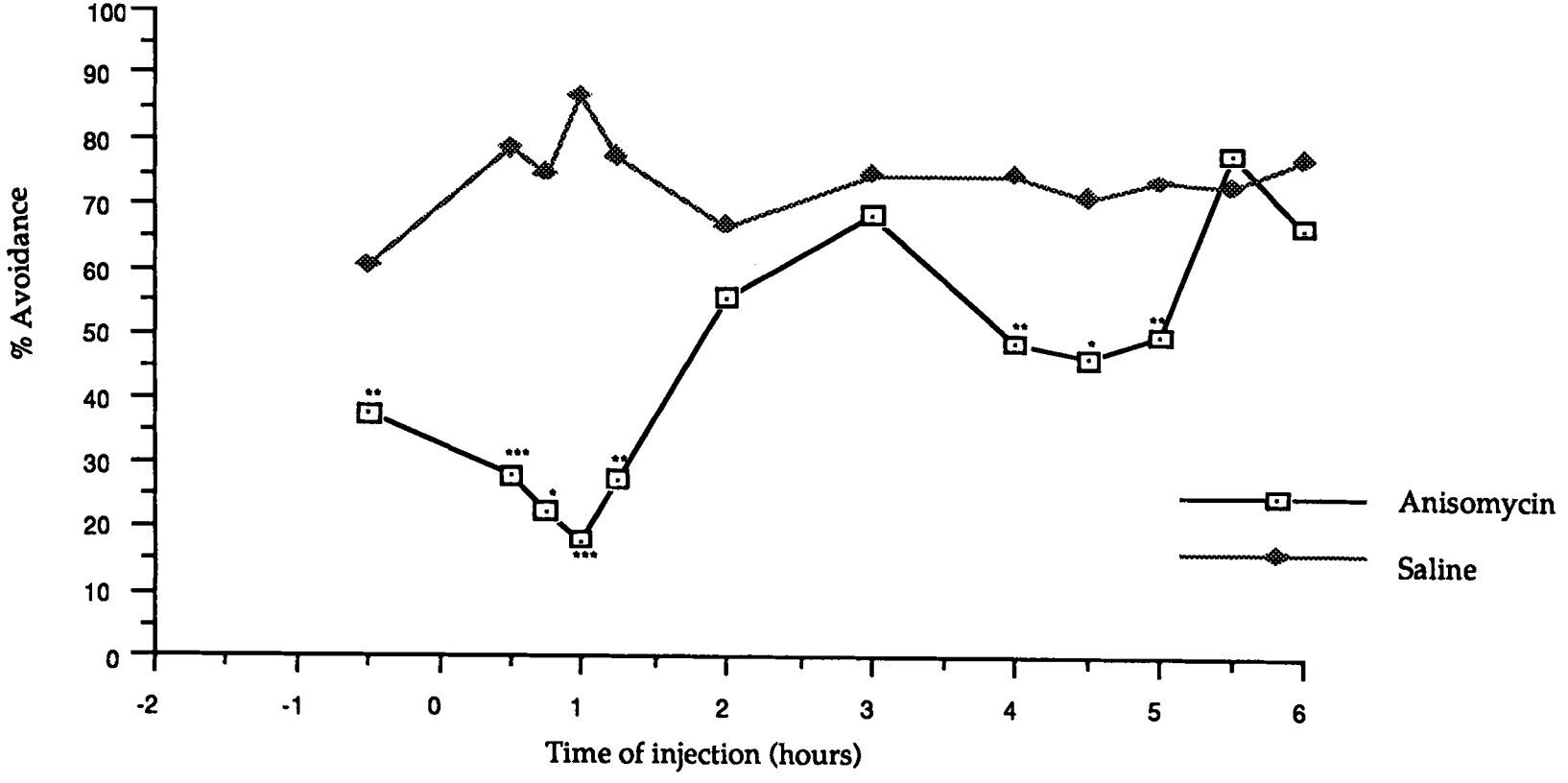
METHOD

Birds were injected with either 0.3 μMoles of ANI in 10 μl of saline per hemisphere or 10 μl saline alone 30 minutes before training, 30, 45, 60, 75 minutes, 2, 3, 4, 4.5, 5, 5.5, or 6 hours after training. Chicks were tested for recall 24 hours later. (n = for ANI, 56, 25, 9, 31, 11, 9, 29, 53, 28, 44, 37, 33; for saline, 61, 26, 8, 23, 9, 9, 28, 52, 28, 46, 41, 36 respectively). Owing to the large numbers of chicks required, one or two time points were performed per batch, and the results for each time point pooled from two to four different batches.

RESULTS

Bilateral injections of ANI resulted in two waves of ANI susceptibility. The first wave was between -30 minutes to +90 minutes, and the second wave 4 to 5 hours post-training. The results of the experiment are presented graphically in Fig. 6.1.

Figure 6.1



The effect of ANI injections at different times after training on recall at 24 hours. Groups of animals received bilateral intracranial injections of 0.3 μMoles of ANI in 10 μl saline, per hemisphere, at intervals from 30 minutes before-training to 6 hours post-training. Animals in the control group received injections of saline at the same time points. Retention was tested at 24 hours. *, $p<0.10$: **, $p<0.05$: ***, $p<0.01$.

Experiment 6.2. Unilateral injections of ANI during the first and second wave of amnesia.

The aim of this experiment was to determine whether protein synthesis during long-term memory formation and storage was lateralised. This was achieved by unilateral injections of ANI during the first or second wave of protein synthesis.

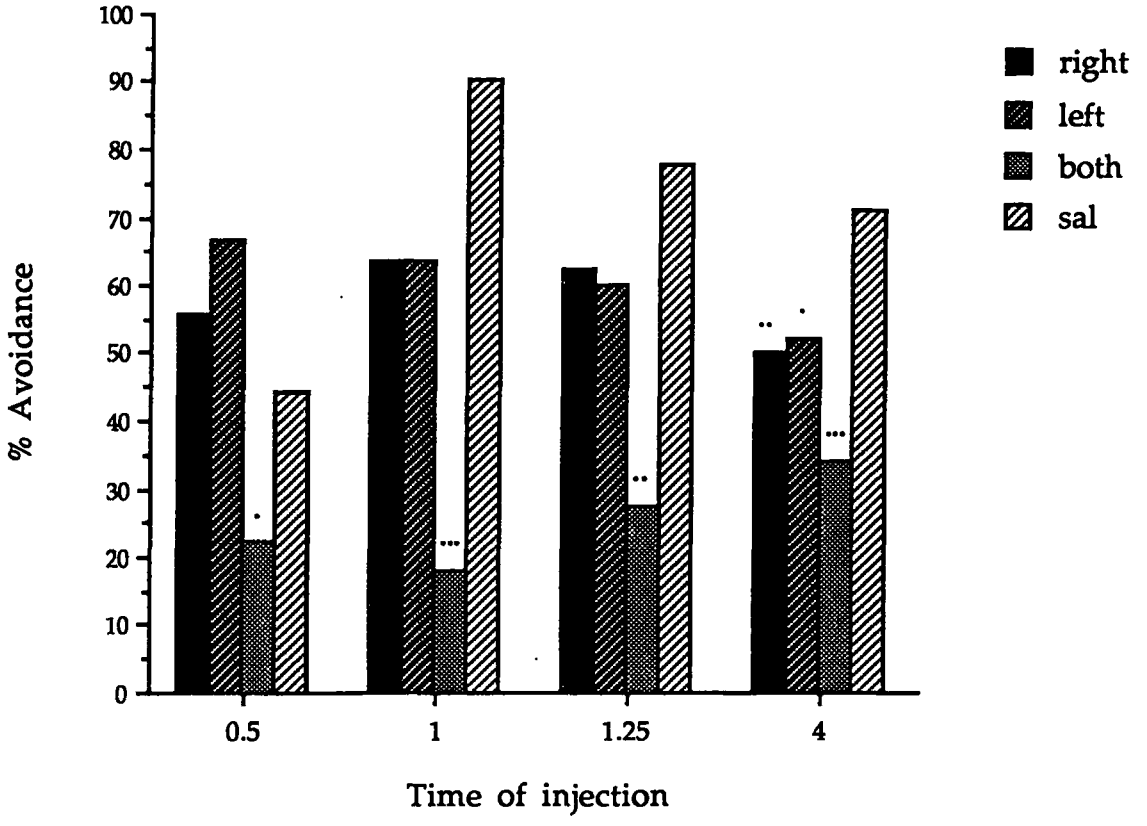
METHOD

On the day of the experiment, animals were randomly assigned to four groups. Test group chicks received injections of 0.3 μMoles of ANI in 10 μl of saline into the left or right hemisphere, and the contralateral hemisphere received 10 μl of saline. Two control groups received either ANI or saline in both hemispheres. For injections during the first observed period of ANI sensitivity, chicks were injected at 30 (n = 9), 60 (n = 10-11), or 75 minutes (n = 8-11) post-training. Because of the large number of animals used, only one time point was tested with one batch of chicks. ANI was injected during the second period of ANI sensitivity at 4 hours post-training only (n = 33-36). The data from 3 batches of chicks was pooled. Again, birds were tested for recall 24 hours later. Statistical comparisons were made between chicks unilaterally injected with ANI and bilateral ANI, and saline injected birds.

RESULTS

Unilateral injections into either hemisphere of ANI 30 - 75 minutes post-training did not result in the chicks being amnesic for the task, even though bilateral injections did cause amnesia. Bilateral and unilateral injection of ANI into the right hemisphere 4 hours post-training did result in amnesia. But, unilateral injections into the left hemisphere did not induce amnesia. The results of the experiment are presented graphically in Fig. 6.2.

Figure 6.2



The effect of unilateral injections of ANI during the first and second wave of protein synthesis on recall at 24 hours. Animals were injected with 0.3 µMoles of ANI in 10 µl saline in either the left or right hemisphere with 10 µl of saline in the contralateral hemisphere. Two control groups were also included, which received 10 µl of either ANI or saline in both hemispheres. Groups of chicks were injected at the times shown. Retention was tested at 24 hours. *, $p<0.10$: **, $p<0.05$: ***, $p<0.01$

Experiment 6.3; The effect of increasing the dose of ANI on unilateral injections during the first wave of protein synthesis.

In this experiment, the concentration of ANI was doubled to 0.6 μMol/10 μl in an attempt to determine whether the drug was rapidly diffusing to the contralateral hemisphere and hence acting at a sub-efficacious concentration during the first wave of protein synthesis. Only the 30 minutes post-training time point was investigated, since injections at this time point appeared to have the most profound effect on inhibition of recall (fig. 6.1).

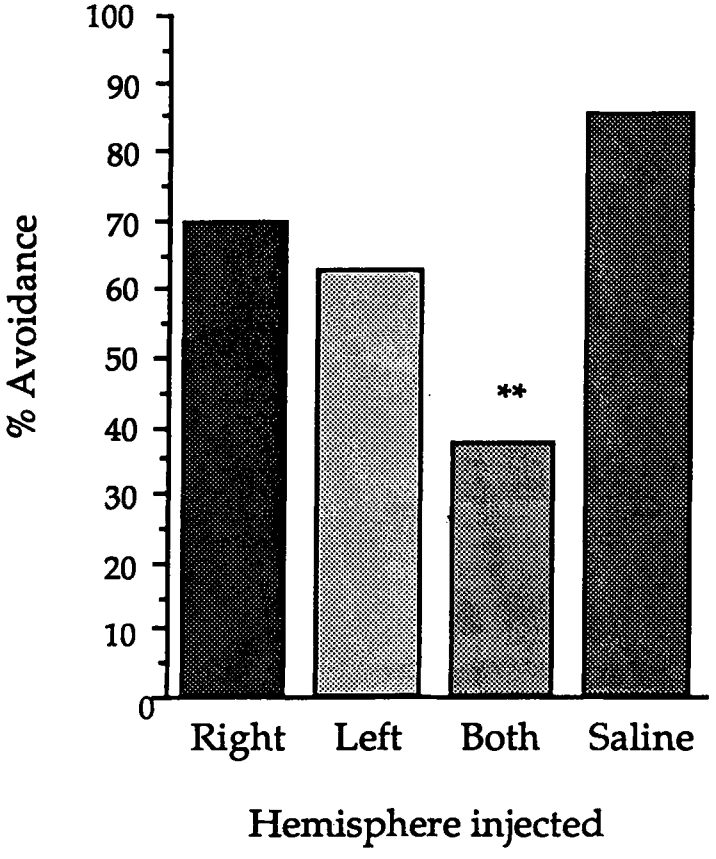
METHOD

The experimental protocol was exactly the same as in experiment 6.2. except that, where appropriate, 0.6 μMol of ANI in 10 μl of saline was injected and only the 30 minutes post-training time point was investigated. N = 11-13.

RESULTS

Unilateral injections of 0.6 μMoles of ANI 30 minutes post-training did not cause amnesia, regardless of the hemisphere injected, even though bilateral injections of the drug were amnestic. The results of the experiment are presented graphically in Fig. 6.3.

Figure 6.3



The effect of unilateral injections of 0.6 μMol of ANI 30 minutes after training on recall. Groups of birds were injected with 0.6 μMoles of ANI in 10 μl saline in either the left or right hemisphere and 10 μl of saline in the contralateral hemisphere. Two control groups were also included, which received either ANI or saline in both hemispheres. Groups of chicks were injected 30 minutes post-training. Retention was tested at 24 hours. **, $p<0.05$

Experiment 6.4; Investigation to determine whether the second wave of protein synthesis precedes or coincides with the second wave or glycoprotein synthesis.

The aim of this experiment was to test whether the second wave of amnesia resulting from inhibition of glycoprotein synthesis, previously described in this laboratory (Scholey et al 1992), is a result of *de novo* glycoprotein synthesis or the modification of existing glycoproteins/proteins. Although behavioural pharmacology cannot conclusively answer this question, it can elucidate whether these two waves are concurrent, or if one precedes the other.

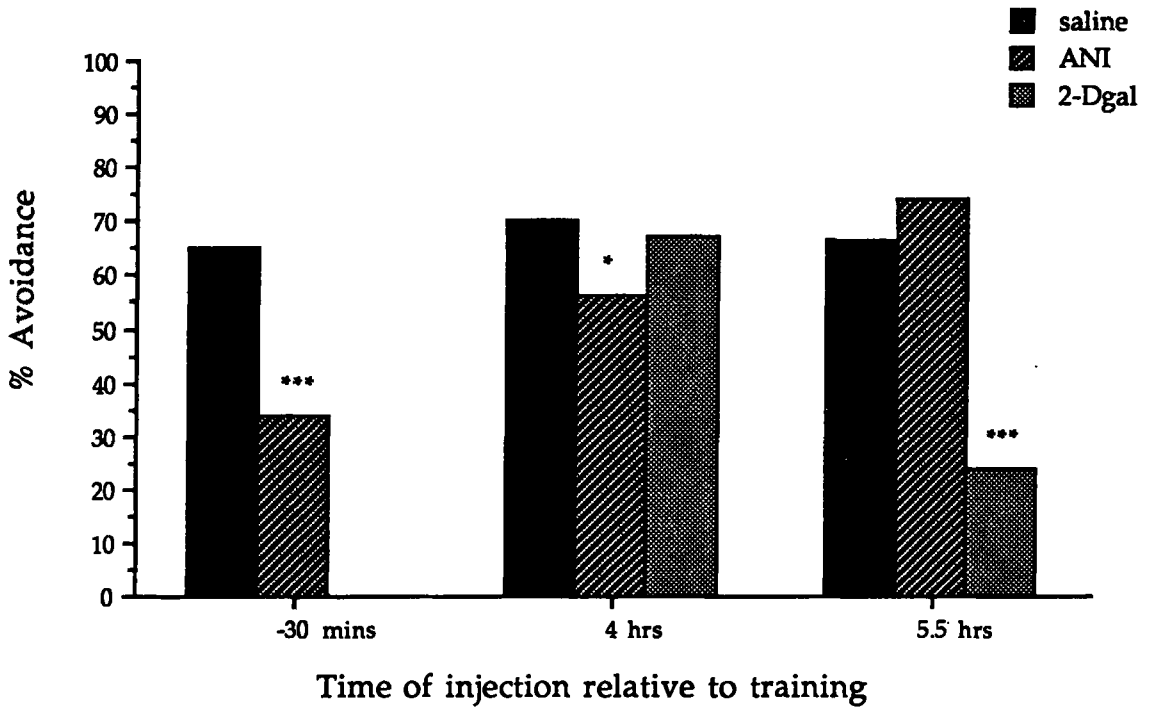
METHOD

Animals were divided into groups. Bilateral intracranial injections (10μl per hemisphere) of either 0.3 μMol of ANI, 10 μMol of 2-Dgal or saline were administered to chicks at either 4 hours (n = 12 for all groups) or 5.5 hours post-training (n = 27, 29, 27 respectively). As a control, to test that ANI was capable of causing amnesia within each batch of chicks, two remaining groups received bilateral intracranial injections of either 0.3 μMol of ANI or saline 30 minutes before training (n = 7 and 8 respectively). Owing to the large number of chicks used, the experiment was split between three batches and the data pooled.

RESULTS

Injections of ANI 30 minutes before- and 4 hours post- training produced amnesia for the task ($p<0.01$; $p<0.05$ respectively), but injection at 5.5 hours after training did not. However, injection of 2-Dgal did not impair recall ability when injected at 4 hours. But, at 5.5 hours, 2-Dgal did produce amnesia ($p<0.01$). The results of the experiment are shown graphically in Fig. 6.4.

Figure 6.4



The effect of injecting ANI or 2-DGal 5.5 hours after training on recall. Bilateral intracranial injections (10µl per hemisphere) of either 0.3 µMol of ANI, 10 µMol of 2-Dgal or saline were administered to groups of chicks at 4 or 5.5hours post-training. Two other groups received either ANI or saline 30 minutes before training. Retention was tested at 24 hours. *p<0.05; ***, p<0.01.

Experiment 6.5; Inhibition of Fos and Jun protein induction 2 hours post-training by injection of ANI 5 minutes after training.

The preceding chapters of this thesis have demonstrated that Fos and Jun proteins are induced in response to passive avoidance training. There remains one important experiment to perform, that is, to test whether ANI, a direct inhibitor of translation, not only causes amnesia but inhibits the induction of these proteins too. The method chosen to study Fos and Jun induction was immunocytochemistry.

METHOD

Chicks were equilibrated over night as described previously (chapter 3). Two-thirds of the birds were trained on the MeA bead, of which half of these received bilateral intracranial injections of 0.3 μMol of ANI in 10 μl per hemisphere, 5 minutes after training. These birds were tested for recall 2 hours later. Only the trained, but uninjected birds that showed recall, the ANI-injected birds that were amnesic and the Quiet controls were then perfused (experiment 3.3). The brains were prepared, immunologically assayed and analysed as previously described (experiment 4.2). N: Quiet = 4; MeA = 4; ANI = 3. Comparisons between MeA birds and ANI injected or Quiet control birds were made using Mann-Whitney-U test.

RESULTS

ANI, when injected 5 minutes post-training, not only caused chicks to be amnesic but abolished the induction of Fos and Jun expression in the LPO and IMHV 2 hours later. The training experience did not induce Fos or Jun expression in the HA (hyperstriatum accessorium), and ANI did not attenuate this relative high level. The results of the experiment are presented graphically in figures 6.6.-6.7.

Figure 6.5. The effect of ANI on training-related Fos induction 2 hours after training. Chicks were trained, of which half received bilateral intracranial injections of 0.3 μMol of ANI in 10 μl of saline per hemisphere 5 minutes later. The animals were tested for recall after 2 hours. Birds that were trained with MeA and showed recall, or ANI injected birds that exhibited amnesia, or Quiet controls, were processed by immunocytochemistry. Results are expressed as the mean number of Fos positive nuclei within a defined region of each area of the brain studied. $^{**}p < 0.025$; $^{*}p < 0.05$

Figure 6.5

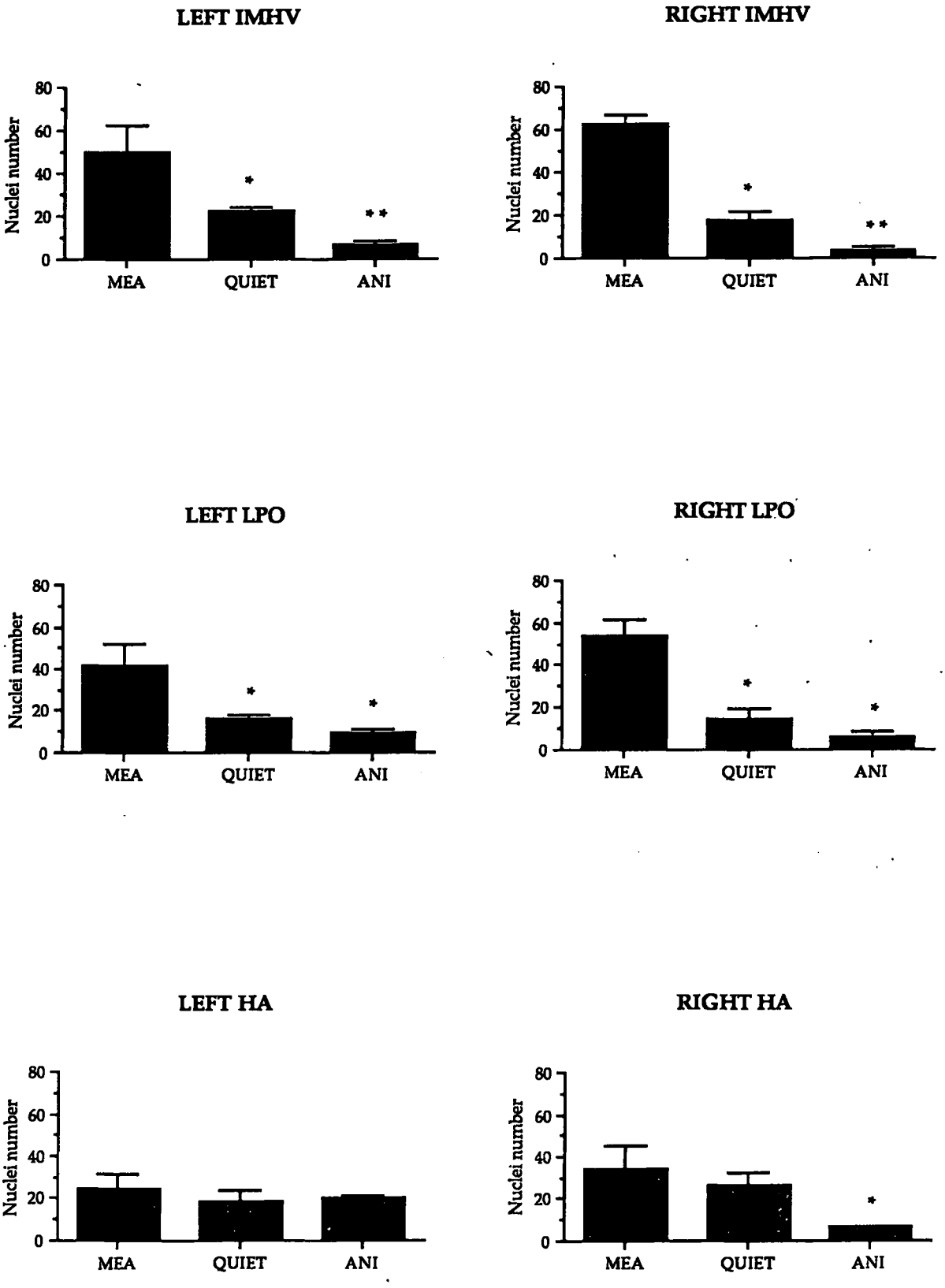
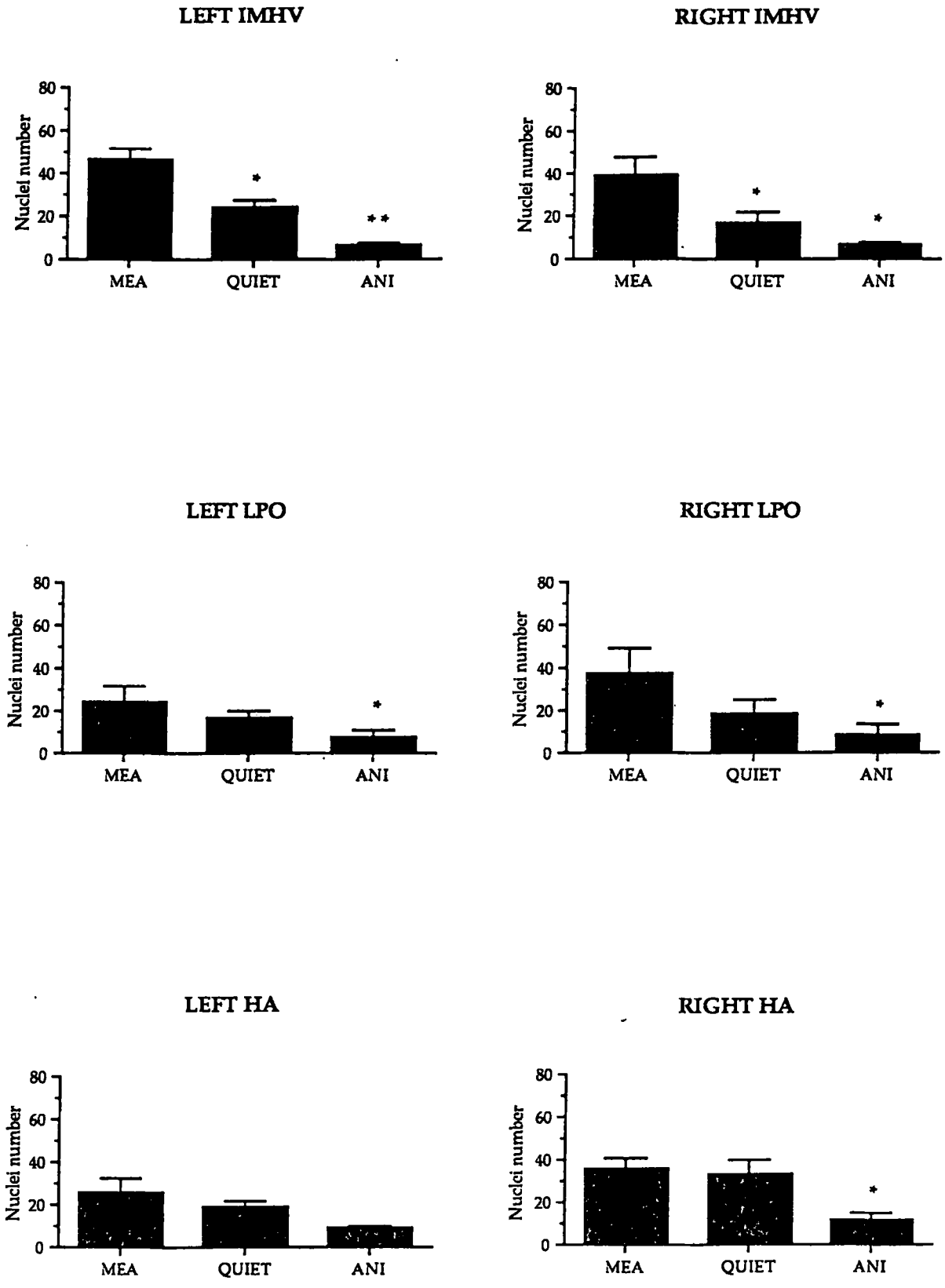

LEFT IMHV
RIGHT IMHV
LEFT LPO
RIGHT LPO
LEFT HA
RIGHT HA
Nuclei number
0
20
40
60
80
MEA
QUIET
ANI
*
**

Figure 6.6. The effect of ANI on training-related Jun induction 2 hours after training. 5 minutes after training 50% of chicks received bilateral intracranial injections of 0.3 μM of ANI in 10 μl of saline per hemisphere. The animals were tested for recall after 2 hours, birds that were trained with MeA and showed recall, or ANI injected birds that exhibited amnesia, or Quiet controls were processed by immunocytochemistry. Results are expressed as the mean number of Jun-positive nuclei within a defined region of each area of the brain studied. $^{**}p < 0.025$; $^{*}p < 0.05$

Figure 6.6


LEFT IMHV
RIGHT IMHV
LEFT LPO
RIGHT LPO
LEFT HA
RIGHT HA
Nuclei number
0
20
40
60
80
MEA
QUIET
ANI
*
**

DISCUSSION

The results demonstrate that there are two waves of sensitivity to ANI. This is interpreted as showing two waves of protein synthesis necessary for the acquisition and retention of a long-term memory in the day-old chick. The first wave occurs from the time of training and is susceptible to ANI for up to 90 minutes. The second wave is of shorter duration and occurs between 4 and 5 hours post-training. The first wave was not lateralized, as unilateral injections did not cause amnesia, regardless of the hemisphere injected. This was not due to rapid diffusion of ANI to sub-efficacious amounts because when the dose was doubled, but injections confined to one hemisphere, no significant deficit in recall was observed. However, during the second wave of ANI susceptibiliy there was hemispheric asymmetry. Unilateral injections into the right hemisphere 4 hours after training resulted in amnesia for the training task. Injections into the left hemisphere also decreased the chick's recall ability, but this was not statistically significant. The effectiveness of ANI as an inhibitor of protein synthesis in this paradigm was also demonstrated by its ability to prevent learning-associated induction of the immediate-early genes Fos and Jun, as shown by immunocytochemistry. This is especially evident in the areas implicated previously with long-term memory formation in the chick ie right LPO and left IMHV (chapter 4). Injection of 2-Dgal 5.5 hours after training produced amnesia for the task after the second wave of ANI susceptability. So it appears that the second wave of neural activity consists of protein synthesis followed by glycoprotein synthesis.

Flood et al (1973) found that ANI caused locomotive defects in mice subjected to a step-through passive avoidance task. Preliminary experiments conducted here have suggested that the effect of ANI on locomotion can be overcome by preparation of the antibiotic in buffer of a suitable pH. Studies conducted in the mouse, after training on a discrimination learning task using

two structural analogues of ANI (3-acetoxy-4-hydroxy-2-[m-bromo-p-methoxybenzyl] pyrrolidine and deacetylanisomycin HCL) showed that although they share the effects of depressing locomotion, they were less potent inhibitors of protein synthesis and do not induce amnesia significantly (Squires and Barone, 1974). Thus, they attributed the amnesic properties of ANI to protein synthesis inhibition rather than its non-specific effects.

ANI's effectiveness as an amnestic agent in the day-old chick following passive avoidance training has previously been studied in this laboratory (Patterson et al., 1989). In the experiment presented here, the dose of ANI used was double that employed by Patterson et al (1989), which was found to have no amnestic effect. This discrepancy could be due to different batches of ANI and/or differences in the manner the injections were administered. Rosenzweig et al., (1991) showed that only a small proportion of intracranially injected drugs actually reach the brain, the majority being pushed back out of the hole produced by the syringe. The speed at which a drug is injected (relatively) could in theory be related to the amount of drug received by the brain. So human error may account for some of these anomalies.

Injections of ANI around the time of training and up to 2 hours later, result in amnesia for learning tasks. These training paradigms include active avoidance or discrimination in the mouse or in the rat (Grecksch et al., 1980; Flood et al., 1973; Squire et al., 1974). The amnesia observed in the experiment reported here was due to inhibition of protein synthesis, and was not a proactive effect of the drug. This is because injections before and up to 90 minutes post-training and between 4 and 5 after training were amnesic, but injections in between these two periods were not. A biphasic response to ANI has also been observed in the rat after a brightness discrimination task (Grecksch and Matthies, 1980), where a second wave of amnesia was observed between 4 and 6 hours post-training. If the proposed site of memory

acquisition in the chick is IMHV, and for storage LPO, then injections into the IMHV means that the drug has a relatively long distance to travel in order to reach the LPO. In addition, Patterson et al (1986) showed that the concentration of ANI they employed (half of the dose used here) was amnestic during the first wave and only inhibited protein synthesis by 35%. These points could explain why, at the dose of ANI employed, the degree of inhibition (% avoidance) observed during the second wave (49%), was higher than that seen during the first wave (22%).

Several investigations in the day-old chick using the one-trial passive avoidance paradigm have demonstrated some brain asymmetries in the acquisition and retention of a memory. Lesion studies have confined these asymmetries initially to the left IMHV, with a rapid redistribution to other regions, notably the right IMHV and LPO (Patterson et al 1990). Behavioural studies, where certain antagonists are injected into one or the other hemisphere, show that pre-training, unilateral injections of an inhibitor of NO (Hölscher 1993), oaubain (a disrupter of intermediate memory formation) (Patterson etal., 1986), the NMDA receptor antagonist, MK-801 (Burchualadze and Rose, 1990) or anisomycin (Patterson et al, 1986) resulted in amnesia for the task, regardless of the hemisphere injected. On the other hand, pre-training injections into the left but not right, hemisphere of glutamate in its role as a disruptor of short-term memory formation (Patterson et al, 1986), 7-ClK which blocks the glycine binding site of the NMDA receptor or inhibitors of PKC, resulted in the chicks being amnesic for the task (Steele and Stewart, 1993; Burchuladze and Rose, 1990). These findings suggest that initially the left hemisphere is needed for acquisition, after which the right hemisphere is rapidly recruited. Since each drug injected has different kinetics, speeds of action will differ. Hence, fast diffusing drugs will reach their 'target' faster, but will 'hang around' at efficacious concentrations for a shorter period of time. Thus, such a drug would in theory reach the left IMHV in time to prevent

acquisition of a memory, but fall to sub-efficacious levels so rapidly that the subsequent activation of the right IMHV by the left IMHV is not preventable.

In the majority of cases, pre-training unilateral injections, including ANI, result in amnesia regardless of the site of injection (Patterson et al, 1986). However, the data reported here show that post-training unilateral injections of ANI during the first wave of susceptibiliy resulted in recall, regardless of the hemisphere injected. It is possible that a unilateral injection 30 minutes post-training is past the critical time for protein synthesis. That is, sufficient time has elapsed for enough protein synthesis of essential proteins to occur in the ANI injected hemisphere coupled with the contralateral hemisphere, which has its full repertoire of proteins synthesised, to retrieve the memory. In addition, since the concentration of ANI employed did not fully inhibit all protein synthesis (Patterson et al., 1986), it may be true that sufficient protein synthesis in the ipsilateral hemisphere had occurred. It must be noted that lesion studies where lateralization is apparent, result in total removal of structures/proteins needed for memory acquisition. Hence, there will not be enough information 'stored in the ipsilateral IMHV' after removal, for recall to occur.

Unilateral injections of ANI during the second wave did result in amnesia. Only injections into the right hemisphere caused a statistically significant reduction in recall of memory. However, careful analysis of the effect of unilateral injections of ANI on recall showed that injections into the left or right hemisphere produced approximately the same recall score (50%; right, 52%; left). Because of these similarities in recall score, it may be that both hemispheres are needed for the retention of a memory. A plausible explanation for these results could be in the way a chick actually processes a memory. During the first weeks of a chick's life there is an asymmetry in the thalamofugal projections: that is, the right eye projects mainly to the left

hemisphere, whereas the left eye is almost only connected to the right hemisphere (Rogers and Anson, 1978; Boxer and Stanford, 1985). This leads to the assumption that all the information received by the one eye goes to the contralateral hemisphere (Sandi et al.,1993). Behavioural studies involving chicks have suggested that the right hemisphere analyses the position of the stimuli in space and the left hemisphere categorizes the stimuli, eg. fear (Andrews, 1991). So, if protein synthesis in one hemisphere is abolished, then only a certain aspect(s) of the memory is lost. Use of information stored in the contralateral hemisphere will still enable recall. These results are consistent with passive avoidance training and IEG induction, where Fos, Jun and AP-1 proteins have been shown to be induced in response to learning (and perhaps novel stimulation) in the right LPO, and novel stimulation in the left LPO 2 hours after training (chapter 4). If the IEG, Fos and Jun are acting as a 'third messenger' system in response to the training paradigm, then activation and expression of late genes could occur any time after their initial induction. Another explanation already mentioned is that the site involved in the second wave of activity is the LPO, this is relatively far away from the IMHV and so diffusion of ANI could be a problem.

In previous studies, ANI has been shown to inhibit the incorporation of radio-labelled leucine into proteins. It also abolishes learning related synaptic changes following chick passive avoidance training (Bradley and Galal, 1987). The results reported here demonstrate that when ANI is injected 5 minutes after training, the memory-related induction of Fos and Jun proteins in the LPO and IMHV 2 hours later are abolished. The right and left HA did not express Fos or Jun in response to the training paradigm (chapter 4). But the densities of Fos and Jun positive nuclei in Quiet and MeA birds were relatively high in the right HA, and the addition of ANI significantly reduced the number of positive nuclei. The density of positive nuclei in the left HA was high, but was not as great as in the right HA. Here, ANI did not

significantly attenuate Jun and Fos expression. These results suggest that these areas are not directly related to memory formation, but do show high levels of Fos and Jun proteins. Immunocytochemistry can only provide information about the number of positive nuclei and not the amount of IEG present within a individual nuclei.

The existence of 2 waves of neural activity has previously been shown in this laboratory. They involve glycoprotein synthesis (Scholey et al., 1993) and enhanced electrical activity (Mason and Rose, 1987; Gigg et al., 1993; 1994). The time-windows for electrical activity and commencement of glycoprotein synthesis concur with each other for both waves, suggesting that NCAM formation and electrical activity are important in synaptic strengthening. Since the second wave of protein synthesis occurs approximately 1 hour before that of the glycoprotein synthesis one can presume that proteins are first synthesised and then, where appropriate, glycosylated. One possible fate of some of these glycoproteins could be in NCAM formation.

Future work to determine whether the LPO and/or surrounding areas are involved in the rentention of a memory could be conducted by injecting ANI straight into the LPO 4 hours after training. This would counteract the problem of ANI being diluted to sub-efficacious quantities after its relatively long journey from IMHV to LPO, hence resolving the question whether both LPO or only the right LPO are involved in memory formation.

CHAPTER 7

General Discussion and Future Directions

The aim of this thesis was to establish a role for the immediate-early genes c-*fos* and c-*jun* in the formation of a long-term memory for the one-trial passive avoidance learning task in the day-old chick. It has already been established that administration of drugs that inhibit *de novo* protein synthesis, such as anisomycin, puromycin and cycloheximide, prevent the formation of a long-term memory in animals such as rats (Flood et al., 1972), mice (Grecksh and Matthies, 1980; Flood et al., 1973) and chicks (Patterson et al., 1986). However, the exact genes whose expression was attenuated by these drugs were, until recently, totally unknown.

Earlier work in this laboratory by Anokhin et al (1990; 1991) had shown that c-*fos* (and c-*jun*) mRNA were induced in response to passive avoidance training and the pebble floor learning task. However, other workers have shown that even though these two genes were transcribed there was no evidence of their translation into proteins (eg. Kiessling et al., 1993). Chapter 2 clearly demonstrated that this was the case, where the MeA training experience induced c-*fos* mRNA but not its protein products. This could have been due to the chicks not recalling the training experience as chicks were not tested for recall in these experiments. Hence, the act of pecking the bead could have been sufficiently severe to induce the messenger but too weak to cause any subsequent translation. This stimulus was different from training chicks on a Water bead as shown in chapter 4, where Fos and Jun proteins were induced in both left and right IMHV and LPO. In this case, one cannot determine whether the chicks were forming an appetitive memory of the Water bead, or have no recall of the bead, but either way they are likely to peck at the bead. However, the salient point derived from chapter 2 is that, if one is going to study the

induction of an IEG, then one must make sure that it is expressed. In addition, knowledge of the species of Fos and Jun induced provides more spatial information about subsequent late gene activation.

In chapter 3, metrazole treatment, which leads indirectly to the release of all types of EAA transmitters and so has the potential to produce all the species of Fos and Jun proteins in chick brain, gave rise to 2 Fos immunoreactive bands that ran at 47 kDa (believed to be Fos) and 54 kDa. Jun also showed two immunoreactive bands that ran at 39 kDa (believed to be Jun) and 54 kDa. Since the p54 band was common to both antibodies it maybe that this band could represent an AP-1 transcription factor. Western blotting and immunocytochemistry showed that these proteins were exclusively located in the nucleus.

In chapter 4 it was demonstrated that both Fos and Jun proteins were induced in the left IMHV in response to the MeA training experience 1 hour after training. The largest increase in Fos and Jun was observed 2 hours after MeA training in the right LPO. These data suggest MeA training leads to activity in the left IMHV and then in the right LPO. Whether this reflects a sequence of events or independent occurrences in different brain regions remains to be determined. Since there is a similar increase in Fos, Jun and their related proteins in right IMHV and left LPO due to either Water or MeA training, the data indicates that these regions were responding to other aspects of the training paradigm, such as bead colour or orientation. Immunocytochemistry showed that the number of immunoreactive neurones in the right LPO 2 hours after training on an MeA bead, had significantly increased compared to Water controls. This demonstrates that the right LPO is expressing a large quantity of Fos and Jun in a large number of neurones. In order to establish whether both the left and right IMHV and left LPO have more neurones active at different times, or if the activity (number of

neurones) the right LPOs increases further at a later time, one should perform a similar time course to the Western blotting experiment, including the 1, 3 and 4 hour time points after training.

The results described in chapter 5 demonstrated that inhibition of the NMDA-receptor by MK-801 also attenuated the learning-related induction of Fos and Jun proteins previously observed in chapter 4. This clearly implicates the ionotropic NMDA receptor in learning and memory formation. Since 7-chlorokyurenate (7-ClK), an antagonist of the glycine-binding site of the NMDA receptor, also causes amnesia for the one-trial passive avoidance task in the chick (Steele and Stewart, 1993) and can cross the blood-brain barrier, future experiments to determine whether it also abolishes learning-related Fos and Jun expression could be conducted. If 7-CK does attenuate induction and expression of IEG, this would strengthen the assumption that the memory formation cascade involves NMDA receptor activation and subsequent induction of Fos and Jun.

Chapter 6 described a series of behavioural pharmacology experiments to investigate the time course of anisomycin (ANI) sensitivity following passive avoidance learning. The results showed 2 distinct waves of amnesia when the antibiotic ANI was injected bilaterally into the chick's IMHV. The first wave occurred between training and 90 minutes post-training; the second wave of amnesia was 4 to 5 hours after training when chicks were tested for recall at 24 hours. Post-training unilateral injection revealed that, during the first wave of protein synthesis, either hemisphere was capable of retaining the memory upon testing 24 hours later. Unilateral injections of ANI during the second wave were less conclusive; injections into the right hemisphere showed a significant decrease in recall (50% avoid), whereas injections into the left hemisphere gave a similar decrease in recall ability (52% avoid). However, this result was not significantly different from saline injected controls. One possible

explanation for this discrepancy may be that the sites of protein synthesis during the two waves of ANI sensitivity are located in different brain regions. It is plausible that the first wave may occur in the IMHV (the site of injection), whereas the second wave could be at a more distant site such as the LPO, where the problem of diffusion could account for the non-significant findings. It would be necessary to test this hypothesis by injecting ANI directly into the LPO at various time points following training, particularly 4-5 hours post-training during the second wave of ANI susceptibility, and to test for amnesia at 24 hours.

Inhibition of glycoprotein synthesis by 2-Dgal suggests that, in the second wave of neuronal activity, proteins are first synthesised and then glycosylated. This is the opposite of what is observed during the first wave, where Crowe et al (1994) showed that existing proteins were glycosylated because the sensitivity to 2-Dgal preceded susceptibility to ANI. Finally, chapter 6 showed that ANI inhibited the learning associated induction of Fos and Jun proteins.

HOW DOES ALL THIS INFORMATION FIT TOGETHER?

A model for the sequence of events occurring in the chick forebrain following passive avoidance training can be suggested from the results presented in this thesis.

Ca^{2+} entry directly and/or indirectly stimulates the induction of *c-fos* and *c-jun*. These mRNAs are then subsequently translated into Fos and Jun proteins. These proteins could dimerise to form a complex that has an apparent molecular weight of 54 kDa. The timing of *c-fos* and *c-jun* induction and ensuing translation cannot be accurately established from the experiments described here but the data suggests a plausible time course. Given that it takes between 30-45 minutes for *c-fos* mRNA levels to peak and presumably for the

rate of translation also to peak, protein production will maximise after eg. 90 minutes (Muller et al., 1984; Curran and Morgan, 1986). This fits in with the observation that ANI induced amnesia only becomes apparent approximately 60 minutes after training (Patterson et al., 1986). The first wave of ANI susceptibility lasts up to 90 minutes after training (Freeman et al., 1994), however, the exact "cut-off" time for susceptibility may occur at any time between 90 and 120 minutes after training. Interpretation of these two sets of data suggest that the duration of (relevant) protein synthesis (mRNA translation) is approximately 1 hour. During this time ANI not only inhibits the translation of immediate early genes as described here, but will also stop late gene expression. As ANI blocks all protein translation and superinduces IEGs (Sassone-Corsi and Verma, 1987), this shows that it is the protein products, and not the mRNAs, that are required for memory formation.

The Fos and Jun data from chapter 4 suggests that this first wave occurs in the left IMHV. This is substantiated by the fact that pre- (and not post-) training lesions of the left IMHV cause amnesia for the learning task (Patterson et al., 1990). Moreover, Gigg et al (1993) observed that maximal electrical bursting in the IMHV occurred from 4-5 hours, and at between 5-8 hours in the LPO, after training.

The levels of Fos and Jun proteins appears to peak in the right LPO at 2 hours post-training. However, since levels were not ascertained between 1 and 2 hours post training, one cannot be certain if levels were in fact greater at some intermediate time point. In this case, some late genes could have been activated and transcribed, and it is these proteins that were susceptible to the amnesic effects of ANI. Whereas some of the Fos and Jun proteins could have attached to the relevant late gene promoters and waited for further "orders" (Szekely et al., 1993) before these genes were induced during the second wave of ANI susceptibility at 4-5 hours post-training. Thus, the right LPO could be a

site for both the first and second waves of anisomycin susceptibility, with the second wave probably not to any great extent occurring in the left IMHV as shown by the data in chapter 4. The right LPO has been implicated in the second wave because there is an increase in neuronal bursting between 5-8 hours after training (Gigg et al., 1994). It is a distinct possibility that the second wave of protein synthesis does not involve Fos or Jun activation but another IEG and/or the direct activation of late/structural genes. However, even if this second wave is Fos or Jun independent, one of its locations could still be the right LPO. It may be that other IEGs are in fact activated in different temporal and spatial manners.

The fate of some of these newly synthesised proteins could be glycoprotein synthesis, specifically NCAM formation. Injections of 2-Dgal, which inhibits fucosylation of glycoproteins, also produced 2 time windows of susceptibility (Scholey et al., 1993). The first wave was shown to commence before protein synthesis (Crowe et al., 1994). The second wave of 2-Dgal susceptibility occurs after protein synthesis (Freeman et al., 1994). This suggests that, in the second wave, *de novo* glycoprotein synthesis occurs with the formation of a different set of glycoprotein to that in the first wave (Scholey et al., 1993; 1994).

The formation and modification of NCAMs is believed to aid synaptic remodelling and therefore strengthens the "memory pathway". Newly synthesised protein (other than glycoproteins) may result in the later morphological changes observed, such as increase spine number and density (Stewart et al., 1987).

FUTURE WORK

This thesis has created more questions than it has answered. So, futher investigation is warranted. A few examples of the work needing to be performed are listed below:-

(1) Immunocytochemistry time course of Fos and Jun induction post-training, comparable to the Western blotting time course experiments.

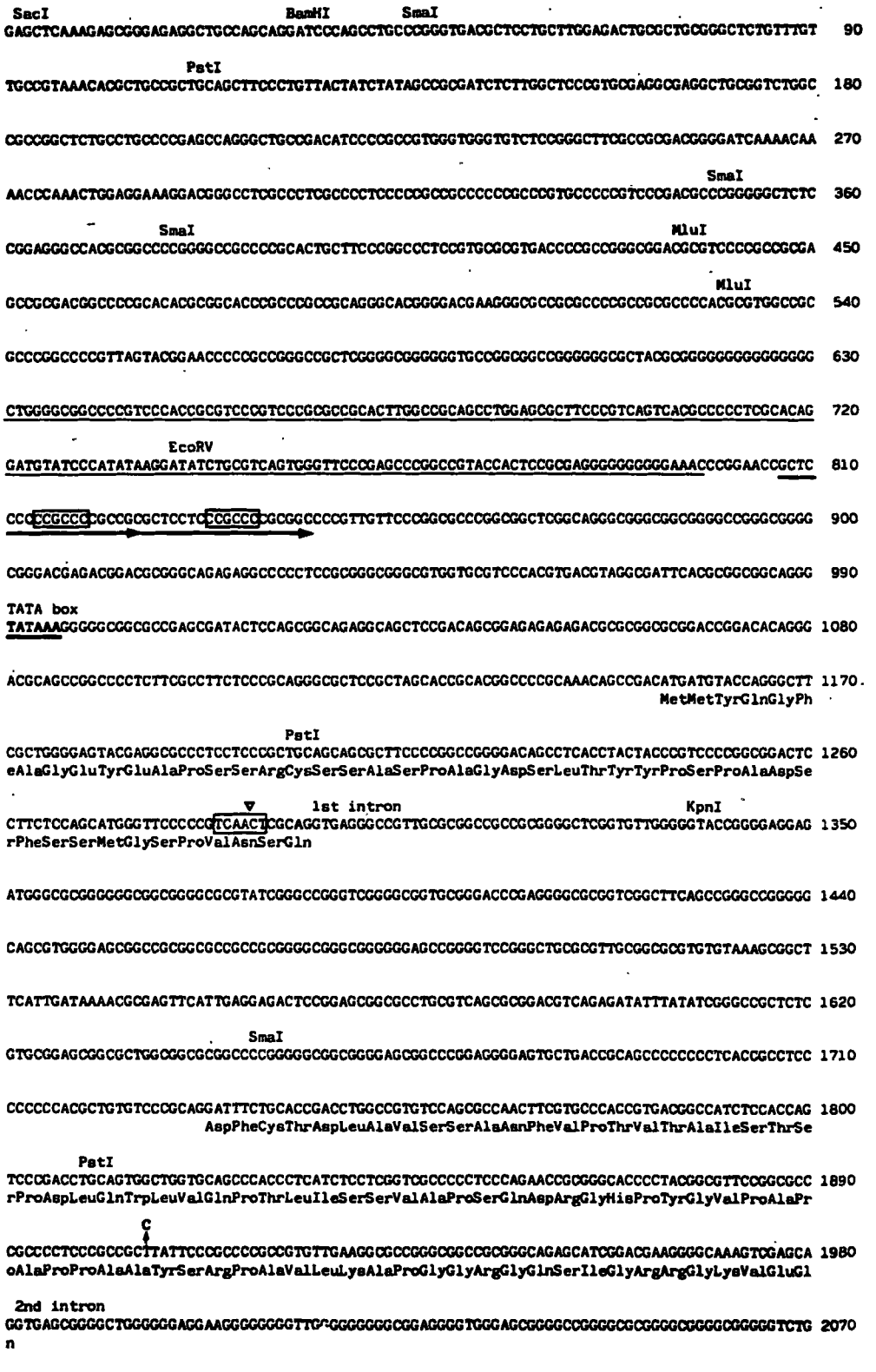
(2) Injections of ANI into the LPO during the second wave of activity to test whether any hemispheric asymmetry occurs.

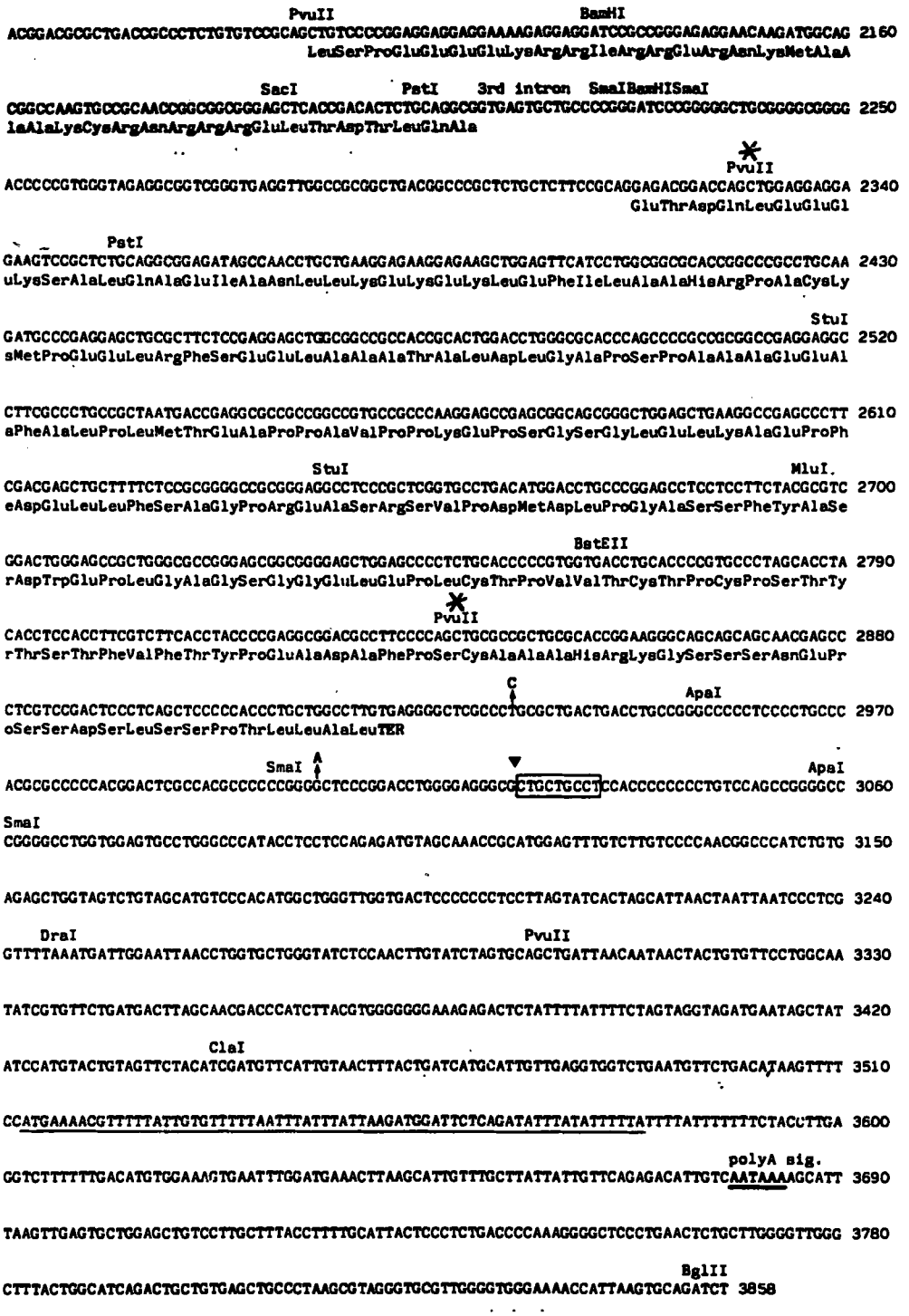
(3) Determination of whether 7-CK attenuates learning-related expression of Fos and Jun proteins.

(4) Using tissue cultured neurones from different areas of the young chick's brain to determine the exact kinetics of IEG's induction via the NMDA receptor. In addition, to identify late gene products whose induction is mediated by the transcription factors such as AP-1 etc, thereby giving an idea of possible protein candidates that are expressed as a consequence of passive avoidance training.

APPENDIX

Structure of c-*fos* probe employed for Northern blotting





* the *Pvu*II-*Pvu*II 508-bp fragment of the exon 4 of chicken c-*fos* gene (Fujiwara et al., 1987)

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